

From the Department of Biosciences and Nutrition
Karolinska Institutet, Stockholm, Sweden

Exploring the Genome-Wide Impact of Estrogen Receptor Alpha and Estrogen Receptor Beta in Breast and Colon Cancer Cells

Karin Edvardsson



**Karolinska
Institutet**

Stockholm 2011

Cover figure by Dr Ron Smith of *Molecular Endocrinology* (2011), 25(6):897-1074, from the article in the same issue by Edvardsson *et al.*, 969–979.

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet. Printed by Universitetservice US-AB, Solna, Sweden

© Karin Edvardsson, 2011
ISBN 978-91-7457-573-6

*In memoriam,
Mormor Ruth*

ABSTRACT

Estrogen signaling is involved in the development and progression of breast cancer and is implicated to be protective in colon cancer. Estrogenic actions are conveyed through transcriptional regulation by ligand stimulated estrogen receptors (ER α and ER β). ER α is upregulated in most breast cancers and is responsible for the proliferative effect of estrogen. ER β on the other hand is usually downregulated, and studies indicate an antiproliferative function. Therapies targeting ER α are available and commonly used in the treatment of breast cancer. In the normal colonic epithelia, however, ER β is the most abundant estrogen receptor and the suggested mediator of the protective effects of estrogen in colon cancer. The role of ER β in breast cancer and colon cancer is not well understood. Thus, exploring the genome-wide impact and contribution of both receptors in estrogen responsive cancers would substantially help to identify novel therapeutic and preventive strategies for these cancers.

In **Paper 1**, we examined differences in transcriptional regulation between ER α and ER β in the breast cancer cell line T47D. We could show that ER β often exhibited an opposing effect on ER α -regulated genes within proliferation and regulation of cell cycle. We also demonstrated a set of genes only regulated by ER β , indicating that, despite the high homology between the two receptors, there are differences in their transcriptional targets. The fact that ER β opposed ER α indicates that ER β activation may be of value in the treatment of breast cancer. To further explore the transcriptional role of ER α in breast cancer, we performed large-scale analyses of microRNA in 24 hours estrogen treated ER α -expressing T47D cells, **Paper II**. However, we found no evidence of direct and rapid regulation of mature miRNAs by ER α .

In **Paper III**, we studied ER β gene regulation in colon cancer cells. We could show that ER β -expressing xenografts grew significantly slower than those lacking ER β . Further we demonstrated that ER β induced a transcriptional response independently of ER α and induced inhibition of the proto-oncogene MYC and other G₁-phase cell cycle genes. In **Paper IV**, we dissected the regulatory networks of ER β -induced transcriptional changes in human colon cancer cells. The set of genes changed by ER β varied in different colon cancer cell lines, however, corresponded to the same biological processes such as cell cycle regulation and kinase activity. In addition, we identified the ER β -driven downregulation of the transcription factor PROX1 as a key mechanism behind a large proportion of the transcriptional changes. In **Paper V**, we studied the effect of long term expression of ER β on the miRNA pool in SW480 colon cancer cells. While we could not show a direct and rapid effect of ER α on the miRNome, we showed that long term expression of ER β did induce large changes in the miRNA pool in colon cancer cells. In particular, we found the oncogenic miR-17-92 cluster to be downregulated and proposed this to be a consequence of the ER β -induced downregulation of MYC.

In conclusion, we have shown that ER β is antiproliferative in breast and colon cancer cells, both when co-expressed with ER α and alone, as well as identified key signaling pathways. We suggest that activation of ER β will have a beneficial effect for treatment or prevention of estrogen dependent cancers.

LIST OF PUBLICATION

- I. Williams C, **Edvardsson K**, Lewandowski S, Ström A, Gustafsson JÅ. *A genome-wide study of the repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells*. *Oncogene* (2008), 27, 1019-1032.
- II. Katchy A, **Edvardsson K**, Aydogdu E, Williams C. *Estradiol-activated estrogen receptor α does not regulate mature microRNAs in T47D breast cancer cells*. *The Journal of Steroid Biochemistry and Molecular Biology* (2011), doi:10.1016/j.jsbmb.2011.10.008.
- III. Hartman J, **Edvardsson K**, Lindberg K, Zhao C, Williams C, Ström A, Gustafsson JÅ. *Tumor Repressive Functions of Estrogen Receptor β in SW480 Colon Cancer Cells*. *Cancer research* (2009), 69, 6100-5106.
- IV. **Edvardsson K**, Ström A, Jonsson P, Gustafsson JÅ, Williams C. *Estrogen Receptor β Induces Antiinflammatory and Antitumorigenic Networks in Colon Cancer Cells*. *Molecular endocrinology* (2011), 25, 969-979.
- V. **Edvardsson K**, Vu HT, Kalasekar SM, Ponten F, Gustafsson JÅ, Williams C. *Estrogen receptor beta expression induces changes in the microRNA pool in human colon cancer cells*. Manuscript.

Related publications not included in thesis:

Williams C, Helguero L, **Edvardsson K**, Haldosén LA, Gustafsson JA. *Gene expression in murine mammary epithelial stem cell-like cells shows similarities to human breast cancer gene expression*. *Breast Cancer Res.* 2009;11(3):R26.

Ström S, **Edvardsson K**, Inzunza J and Williams C. *Estrogen receptor alpha and beta expressed but transcriptional inactive in human pluripotent embryonic stem cells*. Manuscript.

TABLE OF CONTENTS

1	Introduction	1
1.1	Nuclear Receptors.....	1
1.1.1	Structural and functional organization.....	1
1.1.2	A superfamily subdivided into three classes	2
1.2	Estrogen Receptors.....	3
1.2.1	Ligands	3
1.2.2	Transcriptional regulation by estrogen receptors	5
1.3	microRNA.....	7
1.3.1	miRNAs regulate gene transcription.....	7
1.3.2	miRNA biogenesis	8
1.3.3	The role of ER in miRNA regulation	8
1.4	Breast Cancer.....	10
1.4.1	Breast cancer and estrogen.....	10
1.4.2	Treatment of breast cancer	12
1.4.3	Endocrine therapies	12
1.4.4	Endocrine resistance.....	12
1.5	Colon Cancer.....	13
1.5.1	Cause of colorectal cancer	14
1.5.2	The role of estrogen in colon cancer.....	15
1.6	Genome-Wide Techniques to Study Gene Expression	17
1.6.1	Microarray	17
1.6.2	Real-time PCR.....	18
1.6.3	RNA sequencing.....	19
2	Aims of Thesis	20
3	Methodological Considerations	21
3.1	Cell lines	21
3.2	Bioinformatics	21
4	Results and Discussion	23
4.1	Paper I: A genome-wide study of the repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells.....	23
4.2	Paper II: Estradiol-activated estrogen receptor α does not regulate mature microRNAs in T47D breast cancer cells.....	24
4.3	Paper III: Tumor repressive functions of estrogen receptor β in SW480 colon cancer cells	25
4.4	Paper IV: Estrogen receptor β induces antiinflammatory and antitumorigenic networks in colon cancer cells.....	27
4.5	Paper V: Estrogen receptor beta expression induces changes in the miRNA pool in human colon cancer cells.....	30
5	Concluding Remarks and Future Perspectives	32
6	Acknowledgements.....	35
7	References.....	38

LIST OF ABBREVIATIONS

AF-1/2	Activation function-1/2
AI	Aromatase inhibitor
AP1	Activator protein 1/ JUN
APC	Adenomatous polyposis coli
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CD	Crohn's disease
CDH1	E-cadherin
CDK	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
CLU	Clusterin
CRC	Colorectal cancer
DBD	DNA-binding domain
E2	Estradiol
ERE	Estrogen response element
ERK1/2	Extracellular signal-regulated kinase
ER α	Estrogen receptor alpha, NR3A1
ER β	Estrogen receptor beta, NR3A2
FACS	Fluorescence activated cell sorter
FAP	Familial adenomatous polyposis
HAT	Histone acetyltransferases
HDAC	Histone deacetyltransferases
HER2	ERBB2, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
HRT	Hormone replacement therapy
IBD	Inflammatory bowel disease
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LBD	Ligand-binding domain
MAPK	Mitogen activated protein kinase
MCRC	Metastatic colorectal cancer
miRNA	microRNA
mRISC	miRNA-induced silencing complex
MYC	v-myc myelocytomatosis viral oncogene homolog
NCOA3	Nuclear receptor coactivator 3, SRC-3
NCoR	Nuclear receptor corepressor
NR	Nuclear receptor
PR	Progesterone receptor
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
PROX1	Prospero homeobox 1
RE	Response element
SERM	Selective estrogen receptor modulator
SP1	Specificity protein 1
SRC	Steroid receptor coactivator
TSS	Transcription start site
UC	Ulcerative colitis
ZEB1	Zinc finger E-box binding homeobox 1

1 INTRODUCTION

1.1 NUCLEAR RECEPTORS

Nuclear receptors (NR) belong to a class of evolutionary conserved transcription factors. Generally, regulation of gene transcription occurs when a ligand, such as thyroid and steroid hormones or free fatty acids, binds to the receptor thus driving it to undergo conformational change leading to its activation. NRs regulate gene transcription involved in a wide array of biological processes, such as metabolism, development and reproduction. Therefore, deregulation in NR signaling has immense consequences in diseases such as diabetes, obesity, inflammation and cancer.

1.1.1 Structural and functional organization

The NRs share a similar structure containing a N-terminal region, a DNA-binding domain (DBD), a hinge region, a ligand-binding domain (LBD) and a short C-terminal domain (Figure 1) (126).

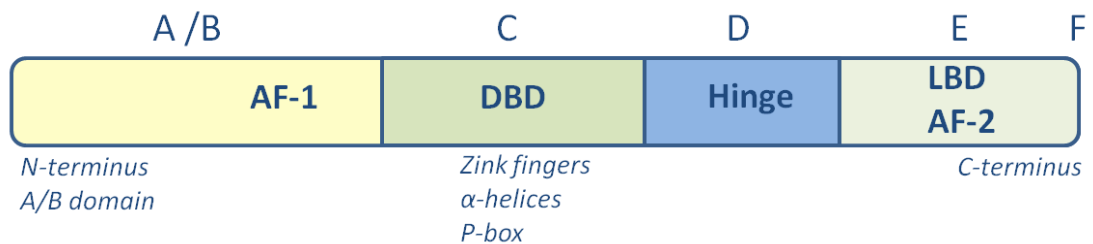


Fig 1. General structural organization of NR consists of five domains named A-F. The A/B domain contains a section important for cofactor interaction, the C domain contains the DNA-binding domain, the D domain contains the hinge connecting DBD with LBD, and is also the target for different post-translational modifications and the E domain contains the ligand binding domain and is also important for cofactor interactions. The function of the F domain is not fully understood.

The N-terminal A/B region varies both in length and sequence between different NRs and contains, for the majority of NRs, a transcriptional activation domain known as AF-1. AF-1 is important for interaction with coregulatory proteins as well as for ligand-independent transcriptional activity. The DBD is responsible for the binding to specific DNA sequences. Several residues, amongst others two zink-finger domains, build up the core of the DBD; two α -helices. The first helix contains the P-box, the residues critical for interaction with the two DNA half sites, while the second α -helix stabilizes the DBD structure (D-box) (4).

Initially, it was believed that the primary purpose of the hinge was to serve as a linker between the DBD and the LBD. However, several studies have now shown that the hinge may change the receptor function through providing interaction surfaces for cofactors and/or post-translational modifications such as SUMOylation (205). The ligand-binding domain contains the ligand-binding pocket and a ligand-dependent

activation function domain (AF-2). AF-2 is necessary for recruitment of a variety of coactivating proteins.

Both the size and shape of the binding-pocket as well as the hydrophobic/hydrophilic nature of the pocket surface correlates with ligand specificity of the receptor (33, 240, 260). The steroid receptors GR, AR, PR, and ER have smaller volumes within their binding pockets compared to other NRs, as well as specific polar side chains of the pocket surface, which provides them with a high affinity towards a small number of ligands. Some of the adopted orphan receptors exhibit a larger volume binding-pocket compared to the steroid receptors, giving them the possibility to interact with a large number of ligands with different structures and of different sizes (159).

1.1.2 A superfamily subdivided into three classes

The NRs can be classified in different ways. Sequence alignment and phylogenetic tree construction reveals six evolutionary groups based solely on sequence homology (92). The NRs can also be divided into three classes based on the type of ligand they bind and/or where they are found in the unliganded state (20). Class I is known as the steroid receptor family, binds to steroids (estrogens, glucocorticoids, progestins, androgens, mineralocorticoids) and includes the estrogen receptor (ER), glucocorticoid receptor (GR), progesterone receptor (PR), androgen receptor (AR) and mineralocorticoid receptor (MR) (169). Class II is known as the thyroid/retinoid family, which binds to non-steroids (thyroid hormone, retinoids, prostaglandines) (20) and includes the thyroid receptor (TR), retinoic acid receptor (RAR), vitamin D receptor (VDR) and peroxisome proliferation-activated receptor (PPAR). The third class is a group of receptors to which no known ligands have been found (true orphans) or just recently been found (adopted orphans).

Generally, unliganded class I receptors can be found in complex with heat shock proteins in the cytoplasm. Upon ligand binding, the receptor undergoes a conformational change leading to dissociation from the heat shock proteins and translocation to the nucleus where the activated receptor can bind to its response element (RE) and activate transcription of a gene. Unliganded receptors from group II are most commonly found associated with corepressors at their RE (in the nucleus) consequently leading to a repression of activation. Ligand binding results in conformational changes leading to dissociation of corepressors, recruitment of coactivators and thereby transcription of a gene (4). Recent studies have shown that unliganded class I receptors shuttles between the cytoplasm and the nucleus (173), however it has not been fully established if they have the same ability as class II receptors by binding to RE in the unliganded form.

Class I receptors bind as homodimers to two hexanucleotide half sites, organized as inverted repeats often upstream of the promoter site, separated by a 3bp spacer. Class II receptors and some of the orphan receptors form heterodimers with the retinoid X receptor (RXR) and bind to two direct repeats of the consensus half site sequence separated by 1-5 bp spacer (169).

1.2 ESTROGEN RECEPTORS

Almost 50 years ago it was discovered that estrogen signaling was mediated through a specific high-affinity receptor (132). This estrogen receptor (ER) was one of the first NRs to be cloned in 1986 (97, 98). A second estrogen receptor was identified ten years later, in 1996, (151) and the two receptors received their names estrogen receptor alpha (ER α ; NR3A1; ESR1) and estrogen receptor beta (ER β ; NR3A2; ESR2), respectively. The two ERs are highly homologous in their DNA binding domain, with approximately 97% identical amino acid sequence, and in their ligand-binding domain where they have 56% similarity. However, they only have 24% identity in the N-terminus where the AF-1 domain is found (61).

The two ERs are found expressed in various tissues and cells throughout the body. However, ER α and ER β exhibit different tissue- and cell-type specific expression, sometimes both may be present in the same tissue but in different cell types. ER α can, for instance, be found in the uterus, kidney, prostate, testes, bone, mammary gland, placenta, ovary, liver, certain regions of the brain and white adipose tissue (53, 61, 245). ER β can be found in the ovary (granulosa cells), mammary gland, testis, colon, prostate, lung, bladder, bone marrow and certain regions of the brain (53, 61, 75, 245).

The ERs are functionally unique amongst class I receptors since they can function both as homodimers as well as heterodimers (200). Both receptors can bind to the DNA through the classical estrogen response element (ERE) GGTCAnnnTGACC, and they are both activated by the ligand 17 β -estradiol. Still, activation of the two receptors triggers different responses. When they are co-expressed, ER β often appears to exhibit an inhibitory action and oppose the effect of ER α (175, 193). In addition, ER homodimers and ER heterodimers give rise to different sets of regulated genes (196, 252). Differences in the LBD and AF-1 domain, and thereby differences in cofactor interactions, can be the reasons to the various responses from the receptors.

1.2.1 Ligands

Estrogen is a steroid hormone and is, as all steroid hormones, derived from cholesterol. Figure 2 illustrates the biosynthesis of estrogens (148, 188). Even though estrogen is considered to be a female sex hormone, it also plays an important role in development of male sex characteristics. Both male ER knock-out mice, and mice lacking the enzyme used in estrogen production have impaired fertility, indicating a role for estrogen in sperm maturation (69, 213).

17 β -estradiol (E2) is a natural non-selective ligand for ER α and ER β and is the most potent ER ligand, followed by the two metabolites estrone (E1) and estriol (E3). The last step in the steroid biosynthesis of estrogen involves aromatization of androgen and testosterone to estrone and estradiol with the enzyme aromatase cytochrome P450 (222). The main source of production of estrogens in the premenopausal woman is the granula cells in the ovaries. In men and in postmenopausal women, estrogen is also produced at extragonadal sites in mesenchymal cells, in adipose tissue, osteoblasts and in the brain. In men, estrogens are also produced in the testis (221, 222).

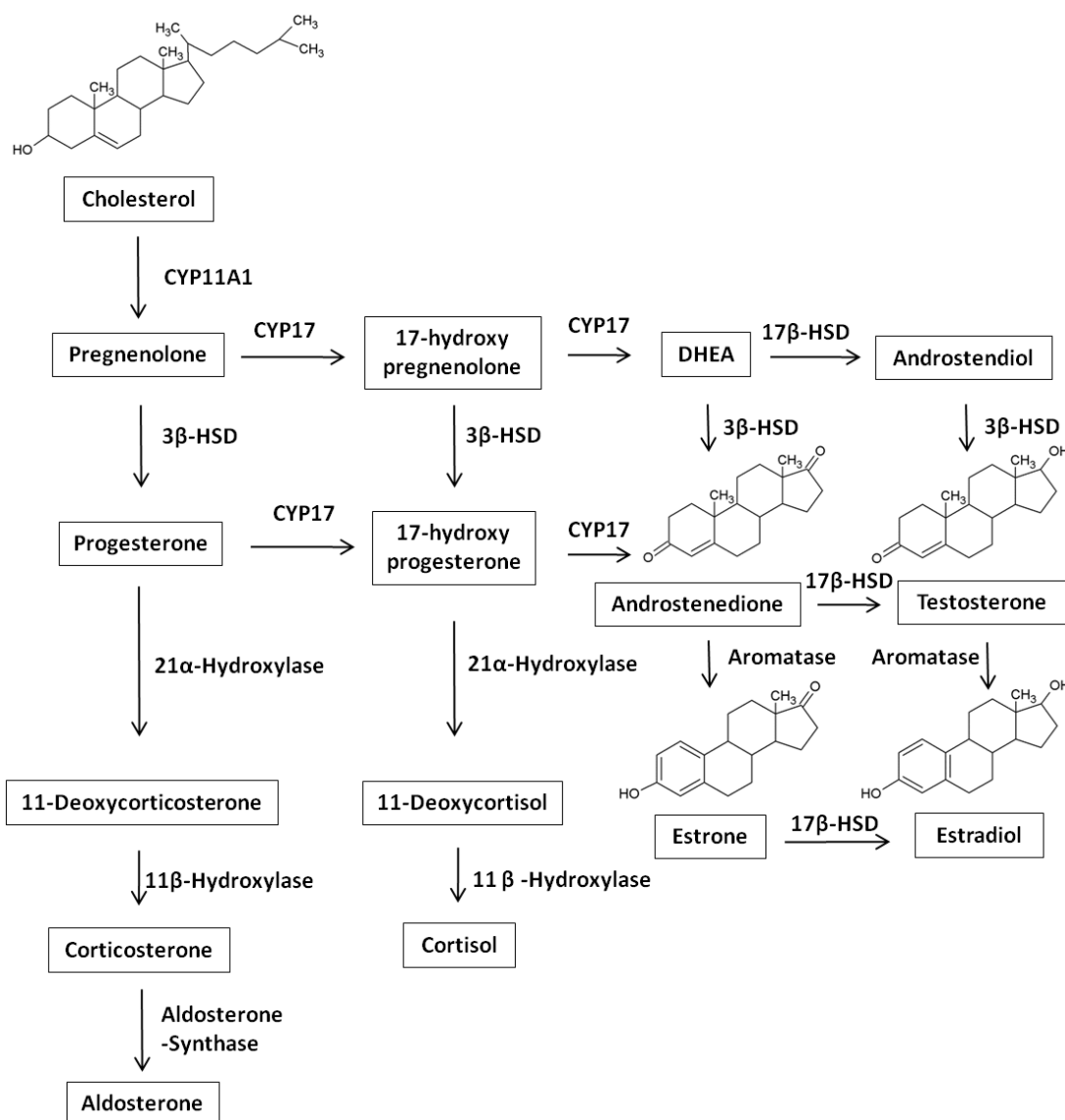


Fig 2. Cholesterol is the first step in steroid biosynthesis. Several enzymes catalyze the different products into different steroids such as glucocorticoids, mineralocorticoids, androgens and estrogens.

In addition to the natural ligands, there are several synthetic compounds able to function as agonist and/or antagonist on the two ERs. For instance, the synthetic compound ICI 182 780 (fulvestrant) is a pure antiestrogen for ER α in the sense that it reduces ER α protein levels in a dose dependent manner (62). Tamoxifen is a selective estrogen receptor modulator (SERM) that acts on both of the ERs but gives different response in various tissues. Its active metabolite, 4-hydroxytamoxifen, is a pure antagonist for ER β and for ER α in breast tissue but an agonist for ER α in bone and uterus (167, 201).

After ligand binding, an important α -helix in the C-terminus, α -helix 12, undergoes a reposition, thereby affecting the ability of AF-2 to bind to coregulators. For example, antagonist bound to ER α will reposition helix 12 so that it overlaps with the coactivator binding sites thus preventing binding of coactivators and consequently prevents transcriptional activity (218). A ligand (agonist or antagonist) may inhibit one or both AF domains. Since the N-terminus AF-1 differs between ER α and ER β , an antagonist-

driven reposition of helix 12 may reduce transcriptional activity differently in the two ERs depending on if helix 12 occludes the coactivator recognition groove or not.

1.2.2 *Transcriptional regulation by estrogen receptors*

Transcriptional regulation by the two estrogen receptors may occur through several different pathways, see Figure 3 (32, 118). The classical estrogen signaling pathway occurs when liganded ER dimerizes and either binds directly to an ERE or to other response elements through tethering to other transcription factors such as AP-1 or SP1. In addition to the genomic regulation, there is also a rapid non-genomic regulation where a signal cascade is activated, following activation of secondary messengers (183). The non-genomic ER activation is too rapid to involve activation by mRNA or synthesis of protein. Instead, this often involves activation of various protein-kinase cascades. An example is ER activation of endothelial nitric oxide synthase through protein kinase-mediated phosphorylation (44). The fourth way of estrogen receptor activation includes ligand-independent posttranslational modifications such as phosphorylation, SUMOylation, methylation and acetylation of the estrogen receptor (19, 92, 138, 233). The different ways of transcriptional regulation by the ERs result in varying outcomes; e.g. different sets of genes can be regulated by liganded ER β and unliganded ER β (252).

The ERE is a *cis*-regulatory element. Whilst it can be found less than 10 kb upstream of estrogen regulated genes, it is estimated that half of all conserved non-coding elements in vertebrates are >250 kb away from their associated genes (242). Theories on how distal binding sites physically can participate in transcriptional regulation suggest that it may be through coregulator recruitment to the target promoters via DNA looping (39, 42). Many estrogen responsive genes have both proximal and distal binding sites (43, 124, 160). Actually, only a small fraction (5%) of ER α binding sites are located < 5 kb upstream of transcription start site (TSS) of the closest gene while 38% maps to intronic regions and 23% are within 100 kb from 5' start site and 19% are within 100 kb of the 3' polyadenylation site (160). This indicates that ER α can regulate transcription through DNA interactions both proximal and distal of the TSS, not limited to orientation (5' or 3'). Most ChIP studies have been performed in ER α -expressing MCF7 cells and since the ERs have high homology in their DNA binding domain it has been assumed that these binding sites also are true for ER β . There are no known ER β -expressing breast cancer cell lines, therefore ER β binding studies have been performed by overexpression of ER β in MCF7 cells. These studies have confirmed that the ER α pattern with distal and proximal binding sites also is true for ER β binding sites, in which only 3% of all ER β binding sites are within 1kb from either end of a gene (259). Further, a substantial overlap of DNA binding sites between the two liganded receptors has been identified (47, 100).

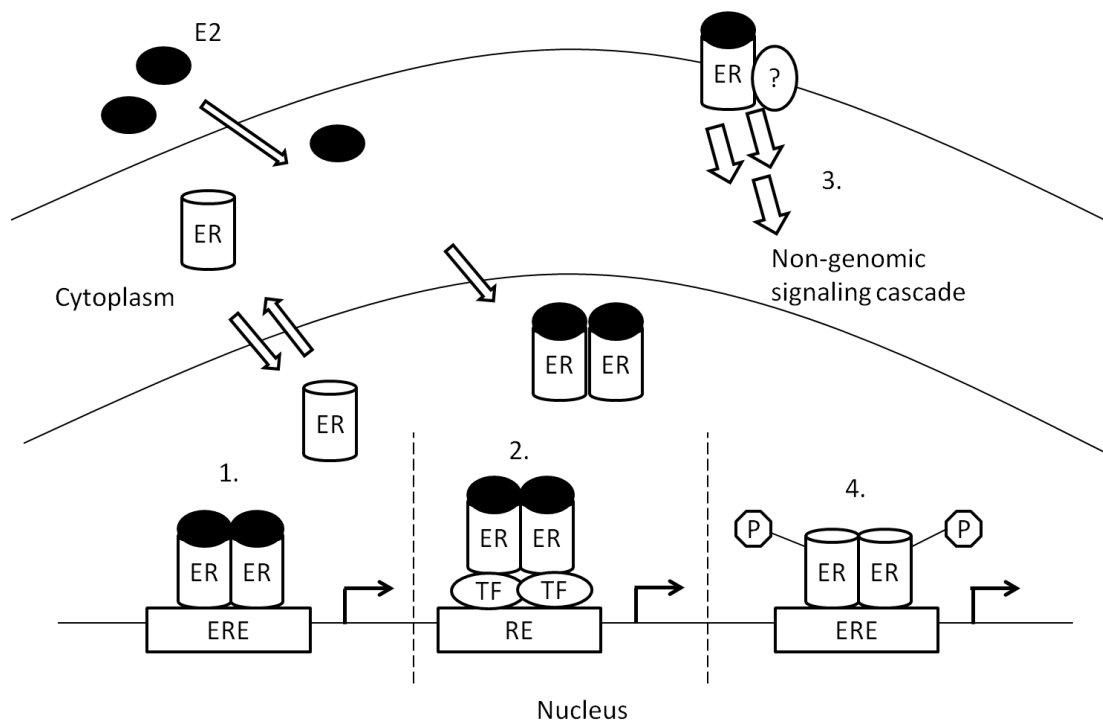


Fig 3. Different mechanisms of ER action. 1, 2 and 4 illustrate the genomic action while 3 illustrates the non-genomic action. 1) Estrogen binds to ER. The receptor dimerizes and binds to estrogen response elements (ERE). 2) Ligand/ER tethers to other transcription factors such as c-Fos/c-Jun which in turn binds to respective response element (AP-1/SP1). 3) Ligand activated membrane bound ER in complex with other factors activates non-genomic signaling cascades. 4) Several different activated cascades lead to phosphorylation of ER which binds to ERE and results in ligand independent transcription.

Transcriptional regulation by the ERs also involves recruitment of coregulators, histone deacetyltransferases (HDACs) and histone acetyltransferases (HATs) (108). Coregulators function as either coactivators or corepressors. Coactivators (e.g. SRC-1, SRC-2, SRC-3) activate transcriptional regulation (141) and the first ones believed to be recruited to activated ERs are the p160 family of proteins and p300 (92). Corepressors (e.g. NCoR, SMRT) on the other hand decrease transcription (153). The coactivators have HAT activity while corepressors mediate HDAC activity (104, 246, 253, 261). NCoR and SMRT does not themselves have enzymatic activities but rather resides in/or recruits transcriptional complexes that contain specific HDACs (92, 111). In addition, the retinoic acid receptor α (RAR α) can associate with the ERs to maintain ER cofactor interactions, although it is not needed for ER recruitment to DNA (214).

ER α transcriptional profiles in breast cancer cells, predominantly MCF7 cells, have been established, with several accepted estrogen stimulated ER α regulated genes such as pS2, SIAH2, GREB1, BCL2 and MYC (45, 89). Even though the ERs have high homology in the DNA binding domain, they still give rise to different transcriptional profiles. This has been illustrated in transcriptional studies in MCF7 cells and U2OS osteosarcoma cells with ER α and ER β expression in which only 25% and 23% of all ER β regulated genes were shared with ER α , respectively (100, 232). One problem when studying ER β transcriptional regulations in cancer cells is that there are no cancer cell lines endogenously expressing significant levels of ER β . The few studies published

on ER β transcriptional regulation have poor correlation, possibly caused by different techniques for introduction of ER β as well as varying expression levels of the protein. Therefore, there is no general accepted transcriptional profile for ER β in neither breast nor colon cancer cells. One study with ER β overexpression in U2OS osteosarcoma cells have suggested that ER β gene regulation can be divided into three classes: class I which is primarily regulated by unliganded ER β , class II which is regulated only with E2 and class III which is regulated both by unliganded and liganded ER β . Interestingly, AP-1 binding sites are more enriched in class I genes whereas ERE, SP1 and NF κ B1 are enriched in class II genes, suggesting that ER β regulates different sets of genes through interaction with different transcription factors and coregulators (252). However, this has not been confirmed in other cell lines and further studies are needed to define and confirm ER β 's transcriptional profile in different cell types.

1.3 microRNA

1.3.1 *miRNAs regulate gene transcription*

MicroRNAs, miRNAs, were discovered about 20 years ago (155, 249), but their gene regulatory properties were not understood until about seven years ago (208). miRNAs are short (19-25 nt) single stranded non-coding RNA molecules. They suppress gene regulation through complementary binding between the miRNA 5' sequence and the 3' UTR of the target mRNA thereby mediating mRNA degradation and/or gene translation suppression. The target section of the mRNA can occasionally be located in the coding region. The miRNA 5' sequence is known as the 'seed' sequence. The seed consists of nucleotides 2-7 and is the most important part needed in miRNA target recognition (26).

The miRNAs are highly conserved amongst species, e.g. the miRNA Let-7 is identical between human, fly and *C. elegans* (198). Some miRNAs, however, are specific to primates only. Many of the primate specific miRNA are expressed in placenta, brain, testis and epididymis, and some are enriched in human embryonic cells. However, most of the primate specific miRNAs are expressed at low levels in adult tissues compared to embryonic cells, indicating a specific role in reproduction and embryonic development of primates (162). Human, mouse, zebrafish and fruit fly all have approximately the same sized genome. This tells us that other factors are important when it comes to the complexity of the animal. Animals with a more simple body plan tend to have a smaller miRNome., e.g. the human genome has 1424 miRNA genes, mouse 720, zebrafish 358 and fruit fly 238 (mirbase.org, October 2011). Taken together, this point towards the fact that miRNAs may work as “fine-tuners” in regulation of gene expression.

There are 1424 miRNA genes in the human genome (mirbase.org), and together they may target up to 60% of all protein-coding genes (91). A miRNA usually has a relatively mild effect on expression of the target gene. However, each miRNA can target several different genes and many of these targets are often in the same pathway (133), thus the total impact of miRNAs on the transcriptome and proteome is significant.

miRNAs can, through their regulation of target mRNAs, be involved in the regulation of many biological processes, e.g. cell differentiation, fat metabolism, apoptosis, stress and cell proliferation (10, 25, 36). Mice with a non-functional miRNA machinery die early with severe developmental defects (30), while knock-out of a specific miRNA only has mild effects. Thus, a functional miRNA machinery is critical in normal biological processes. Consequently, deregulated miRNA expression may lead to several diseases. Altered miRNA levels have been detected in several different cancers, infection, cardiovascular disorders, diabetes, inflammation and autoimmunity (58, 67, 103, 107, 250).

1.3.2 *miRNA biogenesis*

miRNA genes are transcribed from the DNA by RNA polymerase II into primary miRNAs (pri-miRNAs). A pri-miRNA can be several hundreds of nucleotides in length and may contain several imperfect hairpin loops corresponding to several precursor miRNAs (pre-miRNA). The nuclear protein DGCR8 and the enzyme Drosha introduce a cleavage to liberate the hairpin loop from the pri-miRNA. The resulting hairpin, pre-miRNA, is about 70 nucleotides in length and is exported from the nucleus to the cytoplasm. The protein Dicer then cleaves the loop, leaving a double stranded miRNA/miRNA* duplex. One strand of the duplex (passenger or miRNA*) is usually released and degraded while the other strand, the mature miRNA, is incorporated into the Argonaute containing miRNA-induced silencing complex (miRISC), which facilitates the interaction between the mature miRNA and its mRNA target (115). The biogenesis of miRNAs is illustrated in Figure 4.

1.3.3 *The role of ER in miRNA regulation*

ER-induced gene regulation involves transcription by RNA polymerase II. Breast cancer patients expressing ER α have been reported to express a distinct miRNA pattern compared to ER α negative patients (129, 154, 176, 263). Deregulated miRNA has also been found in colon cancer (178, 184, 257). Both breast and colon cancers have been associated with deregulation of ER α /ER β . Taken together, this suggests that the ERs may exert some of their transcriptional effects via regulation of miRNAs.

Most studies have focused on the effect of miRNAs on ER α protein or mRNA levels, such as miR-221/222, miR-206, miR-27a, miR-22 and miR145 (5, 158, 195, 228, 263) and the repressive effect of miR-92 on ER β (7). However, the regulatory effect of ER α and ER β on miRNAs has not thoroughly been examined. So far, there are no studies looking into ER β -driven regulation of miRNAs, and the published studies on ER α regulated miRNAs are somewhat contradictory (31, 248). miRNA-induced downregulation of ER α makes MCF-7 and T47D breast cancer cells more resistant to tamoxifen induced apoptosis (263). This suggests that these miRNAs might be potential targets for improved antiestrogen therapy in breast cancer. It is essential to further explore ER regulation of miRNAs since identification of ER regulated miRNAs might reveal new biomarkers for diagnosis, success of treatment as well as potential targets for novel therapeutics in several different estrogen responsive cancers.

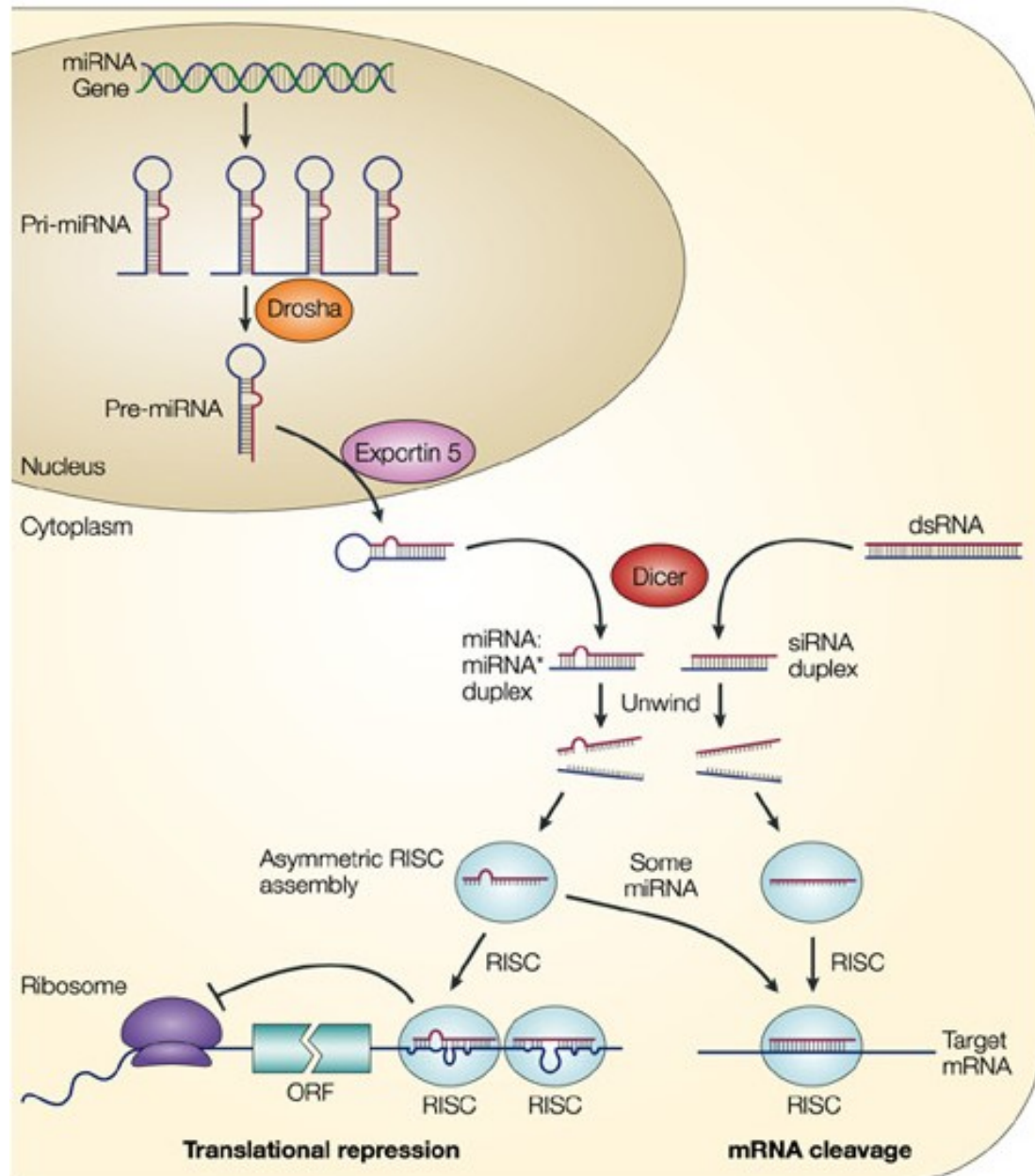


Figure 4. The biogenesis of miRNAs. miRNAs are first transcribed into pri-miRNAs in the nucleus by RNA polymerase II. Drosha cleaves off an imperfect hairpin loop, the pre-miRNA, which then is exported to the cytoplasm where Dicer cleaves off the loop leaving a miRNA/miRNA* duplex. The duplex is separated into single strands and the miRNA associates with the RISC complex and is transported to its target gene resulting in translational repression or degradation. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics (He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation), copyright (2004) (115).

1.4 BREAST CANCER

Cancer is a major health problem in the western world, and trends show that the rest of the world is following in this direction. Breast cancer is the most commonly diagnosed form of cancer in women, and is accountable for 29% of all diagnosed cancer cases (1, 220). Between 15 and 20 women in Sweden are diagnosed with breast cancer every day. This form of cancer is much less common amongst men, and approximately 40 men are diagnosed yearly in Sweden (3).

Most forms of breast cancer are believed to emerge from mutations accumulating during the lifetime. The female sex hormones are involved; use of contraceptives increases the risk for development of breast cancer while pregnancy at young age decreases the risk. 5-10% of all breast cancers are hereditary. Genes that most often are mutated are BRCA1 and BRCA2. Women with one or both of these mutations not only have higher risk of developing both breast and ovarian cancer, but also tend to be affected at an earlier age (13, 177).

In Sweden the incidence of breast cancer has increased with a yearly average of 1.2% during the last two decades (1). The reasons behind this are unclear, but two major contributing factors are the aging population and introduction of mammography screening programs. Screening programs lead to an earlier detection of cancer, and might detect tumors in elderly women that previously would die of other causes before the cancer was progressed enough to be detected. In addition, a change of dietary intake, environmental factors, delay of child birth and the use of hormone replacement therapy (HRT) may also contribute to this increase.

The incidence of breast cancer varies widely between more developed regions such as western/northern/southern Europe, Australia and northern America and less developed regions such as central America, middle/eastern Africa and Asia (81). The incidence per 100,000 women is 19.3 in Eastern Africa and 89.7 in Western Europe. Even though incidence of breast cancer among Japanese women has increased over the past decades, Japan is still considered to be a low-risk country. It has been suggested that the high dietary intake of soy foods significantly has contributed to the low incidence of breast cancer in Asian countries. Soy foods are rich in isoflavones; plant-derived non-steroidal compounds (phytoestrogens) that have estrogen-like properties (6).

1.4.1 *Breast cancer and estrogen*

There are two major epithelial cell lineages in the mammary gland; luminal and myoepithelial. The latter is adjacent to the basal membrane. The major parts of mammary gland development take place during three distinct phases; puberty, pregnancy and lactation, and is under control of growth factors and the steroid hormones estrogen and progesterone (120, 210). A mammary stem cell can self-renew and differentiate into all cell types constituting the mammary gland. Estrogen and progesterone have been shown to regulate mammary stem cells both in number and in ability to propagate into all cell types constituting the mammary gland, despite the lack of expressed ER and PR (18, 135). This indicates that steroid over-stimulation of mammary stem cells increases the risk for breast cancer development. ER α is critical

for regulation of the breast epithelial cell differentiation and proliferation. Females with aromatase deficiency will not develop breasts at puberty unless given estrogen replacement therapy (134). Studies on ER α knock-out mice have shown that ER α is important for normal mammary gland development and is required for development of a normal ductal structure (34, 53, 80). ER β knockout mice showed that ER β is involved in terminal differentiation of the mammary gland but is not involved in ductal growth (86). During menstrual cycle, the epithelial ducts and branches will increase, and during pregnancy and lactation they will differentiate into milk secreting alveoli cells (120, 121). The main ER contributor to the first part is ER α , while ER β has a prominent role in the second one.

ER β is the predominant ER in the human breast. ER α is only expressed in 10-20% of normal resting mammary gland cells (202). In addition to differences in protein expression levels, the distribution of the two receptors differs; in rodent, ER β is found in both epithelial and stromal cells while ER α only is expressed in epithelial cells (48). The ratio between ER α and ER β is changed in breast cancer, 70% of all new breast cancers express ER α , whereas ER β is decreased in advanced stages of breast cancer (76, 114, 194, 197).

Since estrogen plays such an important role in the mammary gland development, it is not surprising that estrogen signaling has a prominent role in breast cancer. Upregulation of ER α results in increased proliferation and subsequent progression of breast cancer. Estrogen treatment of ER α positive breast cancer cells stimulates proliferation while ER β has been shown to suppress ER α transcriptional regulation (105, 113, 183, 230). ER β levels are decreased or lost in breast cancer progression, which may result in a limited inhibitory effect of ER β on ER α -driven proliferation, thus contributing to the pathogenesis of breast cancer.

Most breast cancers, especially in an early stage, are estrogen dependent. This has led to the development of many new therapies targeting the estrogen signaling pathway. It has been stated that breast cancer tumors expressing both ER α and the estrogen regulated progesterone receptor (PR) will benefit the most from endocrine therapy (16, 22, 54). In general, tumors expressing ER α have a better prognosis than ER α negative tumors and are associated with lower-grade tumors, longer disease-free survival and a better overall survival. ER α negative tumors on the other hand have a higher risk for metastases and recurrence (22, 51, 143, 202, 247). It is not entirely clear what role ER β plays in breast cancer. However, ER β KO mice and *in vitro* studies show a connection between decreased or lost levels of ER β and a more invasive phenotype, tamoxifen resistance and overall poor survival (87, 146). However, ER β expression seems to have multiple effects depending on ER α status and invasiveness of the tumor. ER β reduces proliferation in ER α positive cells, yet reintroduction of ER β in more invasive ER α negative breast cancers has the opposite effect and increases cell proliferation (123, 234).

1.4.2 Treatment of breast cancer

Even though the incidence for breast cancer has increased during the last decades, the chance to be cured from this form of cancer has conversely increased. Detection at an earlier stage is one major contributing factor, and also a better understanding of the molecular mechanisms behind breast cancer has led to improved treatments. Breast cancer tumors can be divided into different molecular classes depending on the expression status of ER α , PR and HER2. Tumors status is determined by molecular profiling and therapeutic strategy is then determined based on the molecular class of the tumor. The HER2 protein is amplified in 25-30% of all human primary breast cancers (225), and promotes growth and invasion. VEGF is a protein important to tumors by stimulating formation of new blood vessels. New treatments for breast cancer comprises monoclonal antibodies targeting HER2 and VEGF (e.g. trastuzumab and bevacizumab), and can, at least short term, prevent tumor growth and blood supply to the tumor. In addition, since most breast cancers express ER α , therapies targeting estrogen signaling is widely used in the treatment of breast cancers (tamoxifen, raloxifene and aromatase inhibitors) (114). About 15-20% of all breast cancers have a triple negative profile (ER α , PR and HER2 negative) (59) and these patients have poorer survival compared to hormone responsive subtypes.

1.4.3 Endocrine therapies

Almost 70% of all early breast cancer tumors express ER α , and breast cancer therapies targeting this protein have been widely used for decades (202). Early treatment of estrogen responsive breast cancer included surgery, but in early 1970's a new therapeutic approach was started through to the introduction of the SERM tamoxifen (187). Five years of adjuvant tamoxifen treatment significantly reduces mortality and recurrence for the first 15 and 10 years, respectively (63). Even though tamoxifen gives ER α positive breast cancer patients a better overall survival, there still are some drawbacks. Endocrine treatment with tamoxifen has been shown to be associated with an increased risk of endometrial cancer, blood clots and stroke (83, 84).

ICI 182 780 promotes degradation of the ER α protein and is used as an endocrine treatment to ER α positive breast cancer patients who fail to respond to tamoxifen. Aromatase is the enzyme responsible for conversion of androgens to estrogen. Aromatase inhibitors (AI's) such as anastrozole, exemestane and letrozole are widely used therapies against estrogen responsive breast cancers. They reduce the primary source of estrogen in postmenopausal women, thereby limiting estrogen levels in both plasma and tumors. Studies have shown that advanced breast cancer patients favors from AI's compared to tamoxifen as a first line agent when it comes to overall response rate (88, 187, 259). Tamoxifen is today the most widely used initial therapeutic in early breast cancer, but combination with AI's might improve the overall outcome.

1.4.4 Endocrine resistance

Only approximately 50 to 70 % of all patients with ER α -expressing tumors respond well to hormonal therapy (164, 189). The majority of all patients that initially respond

to tamoxifen develop resistance during treatment (acquired resistance) (128, 211). Also, in some tumors tamoxifen might instead stimulate growth of the tumor. ER α -expressing tumors that are not responding to tamoxifen may be caused by lack of and/or modifications in the allele carrying the gene for the enzyme CYP2D6 (239). This enzyme is responsible for conversion of inactive tamoxifen to its active metabolite; consequently lack of this enzyme will result in lower levels of or no active tamoxifen metabolites. Another explanation to endocrine resistance is the presence of a tamoxifen resistant clone that during time will take over the tumor cell population (52).

Loss of ER α expression, or mutations of the ER α protein, is one known factor contributing to endocrine resistance, although this is only a small fraction of all breast cancers (20%) (190). Instead it has been suggested that post-translational modification of ER α is responsible for most of the resistance. Phosphorylation of ER α results in activation of the receptor independently of estrogen or tamoxifen (41, 181, 223, 244). Activation of alternative pathways that can stimulate proliferation and survival are involved in endocrine resistance. Resistance can be achieved through crosstalk and modulation between these pathways, such as growth factors and kinase pathways that phosphorylate ER (17, 174). In addition, these pathways can during endocrine treatment develop into drivers of tumor growth independent of ER. Some pathways involve HER tyrosine kinase receptor family (e.g. HER2 and EGFR), fibroblast growth factor and stress-related kinases (190). HER2 regulates ER α expression and activity which in turn stimulates phosphatidylinositol 3-kinase (PI3-K)/Akt signaling (74, 229). This ligand-independent activation of ER α results in ER α genomic functions regardless of hormone, thus leading to ER α -induced proliferation independent of tamoxifen treatment (15, 50, 219). ER+/HER2+ breast cancer tumors might therefore benefit from a combined treatment of tamoxifen and inhibitor of EGFR, HER2 and VEGFR (74, 77). Further, reduced levels of ER β might be involved in resistance to tamoxifen treatment (122, 163). However, other studies suggest that tamoxifen has a negative effect in ER β -expressing breast cancers, by antagonizing ER β activated growth-inhibitory genes (157). Therefore, the mechanism behind the potential involvement of ER β in endocrine resistance is not fully understood, and more research is needed.

1.5 COLON CANCER

Colorectal cancer (CRC) is the third most common form of cancer amongst both men and women. Colon cancer corresponds to almost 7% of all Swedish cancer cases reported to the cancer registry in 2009 (1). Equivalent number for men and women in the world is 10% and 9.4%, respectively (81). CRC is a disease more common in developed regions, covering almost 60% of all CRC cases in the world (81). Each year about 608,000 persons die from colon cancer worldwide, accounting for 8% of all cancer deaths. This numbers makes it the fourth most common cause of death from cancer. Just as breast cancer, colon cancer incidences in Sweden have been steadily increasing during the last decade with an average yearly increase of 1.7 % for women and 1.2% for men (1). Each year, about 2,000 men and 2,000 women are diagnosed with colon cancer in Sweden. Also, if rectum and anus cancer were included the number would increase to 3,200 and 2,900, respectively (1). Risk factors

for developing colorectal cancer include smoking, diet, age, alcohol intake, sex, genetic background and intake of hormonal replacement treatment.

1.5.1 Cause of colorectal cancer

Colorectal cancer is caused by uncontrolled growth of the epithelial cells lining the colon. It starts as transformation from normal colonic epithelia into benign adenomatous polyps which may develop into an advanced adenoma and eventually into invasive cancer (170). Development of sporadic CRC is a long multistep process involving several different mutations and requires years before it fully develops into cancer. Mutations include different tumor suppressors and oncogenes, and one of the earliest events in colorectal carcinoma is the inactivating mutation of both alleles of the adenomatous polyposis coli (APC) gene. The APC protein is found mutated in 50% and 80% of all colorectal adenomas and carcinomas (142). This mutation can either be acquired, or inherited. APC was first described 20 years ago through its association with an inherited form of colorectal cancer known as familial adenomatous polyposis (FAP) (35, 101, 136). FAP is an inherited syndrome characterized by an early onset of multiple adenomatous polyps of the colon, and a high risk of developing colorectal carcinoma (96, 136).

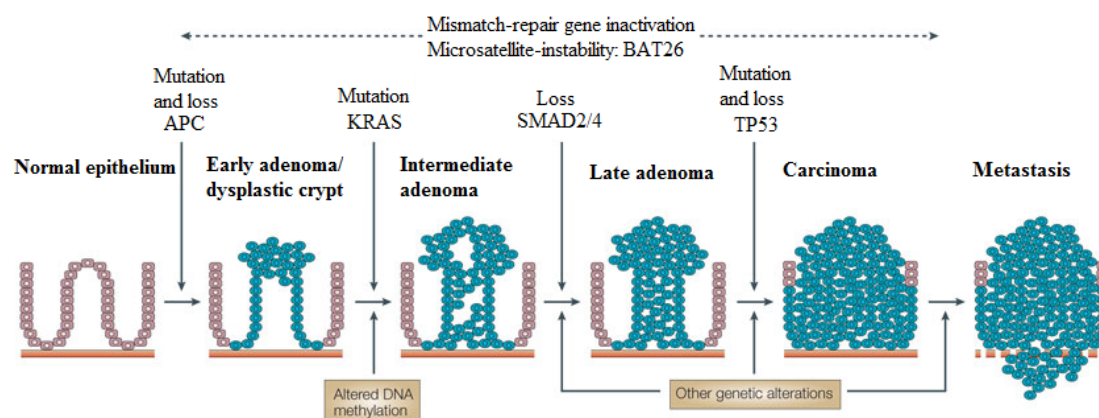


Figure 5. Development of spontaneous CRC from normal epithelium to metastatic carcinoma is a process that takes many years, and requires several steps with gene mutations and/or gene loss. Removal of early polyps may prevent the progression to carcinoma. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Davies RJ, Miller R, Coleman N, Colorectal cancer screening: prospects for molecular stool analysis), copyright (2005) (64).

Most mutations of APC are nonsense mutations resulting in a truncated, non-functional APC protein (101). APC is a major binding partner and regulator of β -catenin in the β -catenin-dependent Wnt signaling pathway (14, 203). Loss of APC will lead to an accumulation of the β -catenin protein which in turn will activate genes important in stem cell renewal and differentiation. When overexpressed, these genes will contribute to cancer development. The impact of accumulating β -catenin, by the non-functional APC gene, was further demonstrated *in vivo* where β -catenin knock-in mice showed formation of multiple polyps morphologically similar to those found in Apc-knock-out mice (109, 191). Kinzler and Vogelstein suggest that APC acts as a “gatekeeper” of colonic epithelial cell proliferation (142). The function of a gatekeeper gene is to keep a constant cell number in renewing cell populations. A mutation in such gene will result

in imbalance between cell division and cell growth. Some colorectal cancers have instead a mutated β -catenin pathway leading to its overexpression due to blocked degradation or mutations in genes responsible for β -catenin regulation such as AXIN1, AXIN2, TCF7L2 or NKD1 (57, 217).

Activating mutations of KRAS and BRAF are other steps involved in transformation and progression of CRC. Both these mutations will lead to constitutive activation of the RAS/MAPK pathway (28). KRAS is a signal-transduction molecule that stimulates cell proliferation, hence an active mutation of this gene will lead to uncontrolled cell proliferation (11). Patients with KRAS mutations are more likely to overexpress p53 and 95% of these adenomas are classified as advanced (71). Mutations of KRAS are not seen in smaller adenomas, and is only seen in 37.7% of colorectal carcinomas and advanced adenomas (11).

In CRCs, inactivation mutations of SMAD2 and SMAD4 are found in 5% and 10-15%, respectively. In addition, it has recently been suggested that inactivation mutations can be found in the SMAD3 gene in 5% of CRCs (78). SMAD2 and SMAD3 are important mediators of the TGF β signalling pathway and form, after activation, a complex with SMAD4. The complex translocates to the nucleus where it together with coregulators regulates transcription (117).

More mutations besides deregulation of the APC- β -catenin-Wnt signaling pathway, KRAS and SMAD2/4 are needed for the cell to transform from an adenoma into an invasive carcinoma. One late mutation is in the TP53 gene coding for the tumor suppressor p53, which is responsible for monitoring cell division and cell cycle. The mutation of p53 results in loss of the wild-type protein, and gain of a missense protein. The missense p53 has been suggested to contribute to decreased apoptosis, increased tumor angiogenesis and affect tumor cell proliferation (78). p53 mutations are not seen in adenomas, but occur in later stages of colorectal carcinogenesis and can be detected in 50-70% of all CRC (79). Mutations of other parts of the p53 regulatory pathway, such as PUMA, p21 and BAX, may also be involved in the transformation to colorectal carcinoma (256). Figure 5 illustrates the progression of normal colonic epithelium to metastatic colonic cancer, and the mutations involved.

Most CRC are either inherited (10-30%) or sporadic (65-85%), but some are also caused by chronic inflammation (180). Patients with any of the two major forms of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC), have an increased risk of developing colorectal cancer (72, 152). The mechanism behind this is not fully understood, but release of several cytokines (IL-6, TNF- α) during chronic colitis and low expression of immunosuppressive cytokines (TGF- β and IL-10) have been shown to promote tumor growth (27, 29, 149, 204, 212, 231).

1.5.2 The role of estrogen in colon cancer

Low intake in fruit and vegetables and a high intake in red and processed meat have been associated with elevated risks of developing CRC (55, 241). However, the impact of diet on initiation and development of colon cancer is being debated. In the last few

years several prospective epidemiological studies have demonstrated that diet might not be as important as previously thought (8, 9, 147, 171).

Since diet might not be as strongly associated with CRC, other etiological factors might be more important in the initiation and development of CRC. The gender distribution in CRC is quite even, although slightly more men than women are diagnosed (81). Interestingly, women have a later onset than men; median age for men to develop CRC is 63 while it for women is 73 years (37). After menopause a woman's body produces less of the sex hormones estrogen and progesterone. For most women, this happens between the age of 45 to 50 (95). A screening of 52,882 US patients with metastatic colorectal cancer (MCRC) from the Surveillance, Epidemiology and End Results registry revealed that younger women (18-44 years old) with MCRC had a better overall survival compared to men of the same age. This was however reversed at older age (>55 years old) where females diagnosed with MCRC lived shorter than men of the same age (119). These results supported previously published data from a study with 2,050 CRC patients that demonstrated an opposing effect of gender on the overall survival at either side of the age of 50 years (145).

Several studies have shown an association between postmenopausal hormone replacement therapy (HRT) and a reduced incidence of CRC (102, 130, 165, 182). The Women's Health Initiative trial was a large randomized trial designed to study the effect of hormone replacement therapy with estrogen and progestin in postmenopausal women (2). Although the trial was terminated due to an increased risk of developing breast cancer for the group receiving the HTR compared to the placebo group, a reduced risk for colon cancer amongst the group receiving the HTR was shown (49). There has also been several reports on an inverse association between oral contraceptives and CRC (161). This indicates that hormonal status seems to play an important role in the development of colorectal cancer. This has been supported in several *in vivo* and *in vitro* studies with estrogen or phytoestrogens: Distribution of estrogen to ovariectomized rats reduced the number of chemically induced tumors in the colon (227). Phytoestrogens are weak ligands to the ER with chemopreventive properties against estrogen related cancers such as breast cancer and colon cancer (150, 179, 235). Treatment with a dietary fiber that by colonic bacterial enzymes is converted to a phytoestrogen, counteracted the intestinal tumorigenesis in ApcMin/+ mice (24) and treatment with phytoestrogen from soy resulted in suppressed colon tumor growth in male rats (207). Further, one small pilot study with five FAP patients receiving a combination of the phytoestrogens curcumin and quercetin showed reduced size and number of adenomas after treatment (56). All this support that estrogen has a protective role in the development of CRC.

In the normal colonic epithelia, ER β is the most common form of the two estrogen receptors (40, 82, 139, 226, 255). ER β expression decreases in the progression of normal colonic tissue to colon cancer. This decrease of ER β expression directly correlates to apoptosis, correlates to the differentiation grade of the tumor and inversely correlates with cell proliferation (23, 85, 131, 144, 185). This suggests that ER β is the mediator of the protective effect of estrogen in CRC. This has further been supported in *in vitro* and *in vivo* studies: ER β overexpression in HCT8 colon cancer cells lead to decreased cell proliferation (172). Treatment with an ER β specific agonist,

diarylpropionitrile (DPN), in mice that spontaneously develop intestinal adenomas (ApcMin/+) resulted in reduced intestinal tumorigenesis (93), and deletion of ER β in these mice had the opposite effect increasing both number and size of adenomas (94). It has also been demonstrated that estrogen stimulated ER β reduced cell growth in nontransformed colonocytes, implying a role for ER β in the protection of the colonocytes from malignant transformation (243). All together, this supports the role of ER β in colon cancer; both as a protector against CRC initiation as well as inducer of decreased proliferation in already transformed colon cancer cells. However, the mechanisms behind this are still rather unexplored. To further establish ER β 's role as a target for CRC prevention or therapy, more studies are needed, both *in vitro*, *in vivo* and in the clinic using ER β specific ligands. In addition, a better understanding of gene pathways activated by ER β will help to further dissect the impact of ER β in CRC and might serve as a tool to improve diagnostics, adapt and select proper therapeutic treatments and help to predict clinical outcome of CRC patients.

1.6 GENOME-WIDE TECHNIQUES TO STUDY GENE EXPRESSION

The focus in this thesis has been to explore genome-wide effects of the estrogen receptors. There are several different techniques that can be applied and below are short explanations of a few of them.

1.6.1 Microarray

The microarray technology has been widely used in genome-wide gene expression studies since the late 1990s (215, 216). This technique offers an easy and rather cheap method to study differences in gene expression between samples. The basics behind microarrays are deposition of DNA corresponding to the full transcriptome, where each gene is represented by one or several probes, on an array. A sample cDNA, labeled with fluorescent dyes, is hybridized onto the probes. The fluorescent intensity of each probe corresponds to the abundance of that gene transcript in the sample. Independent of platform used, microarray data needs to be confirmed with other techniques such as real-time PCR. This is especially true for low abundant genes.

Several different companies offer varying techniques and manufacturing of microarrays. Affymetrix is one of the most commonly used microarray platforms (73). Probes on the GeneChip from Affymetrix contain 25-mer oligonucleotides, synthesized directly on the microarray surface, where the probe sequence is built one base per cycle. Each gene is represented by several different oligonucleotides known as a probe set, both perfectly matched to the target transcript as well as mismatched sequences (73). Specific binding is determined through the signal difference between matched and mismatched transcripts in a probe set. One sample is hybridized per array, meaning that a comparison between two samples requires two arrays. While Affymetrix uses photochemical synthesis to print arrays with short oligonucleotides, Agilent offers printed arrays with 60-mer oligonucleotides using InkJet technology. In addition to offering single-color arrays, Agilent also provides dual-color arrays where the two samples to be compared are hybridized on the same array slide. Another technique is the spotted array where (25-70 mere) pre-synthesized oligonucleotide probes are

attached onto the glass array. These probes can be produced “in-house” of academic laboratories or companies, or can be bought as ready-made collections of oligonucleotides (e.g. Operon’s 35k 70mer library covering all known human genes). Spotted arrays are usually two-channelled which means that each of the two samples are labeled with different fluorescent dyes and hybridized onto the same array. A different technique is offered by Illumina, where the oligonucleotides are attached to small beads instead of a glass slide. One benefit of Illumina is the possibility to process several samples in parallel.

The major benefit of printed arrays is the possibility to add more probes onto an array that what mechanically is possible for spotted arrays. Spotted arrays have the capacity to cover all known genes. However, the benefit with more probes, as offered by printed arrays, is the possibility to add replicates as well as splice variants. Both Affymetrix and Illumina offer multiple replicates for each gene, thereby increasing the possibility to detect differentiated genes despite of one bad probe. The drawback of Illumina compared to Affymetrix is that each transcript is detected with multiple probes with the same sequence instead of multiple probes with different sequences (73). Benefits of spotted arrays are the cheaper price and the possibility to customize the array for each experiment. However, results from custom made spotted arrays may not be so easily compared to results from commercially printed arrays due to reduced sensitivity, reduced printing efficiencies and reduced quality with missing spots (21, 73). Commercially available printed arrays have been quite expensive, but during the last years, both Affymetrix and Agilent arrays have drastically dropped in price. Still, purchasing spotted arrays are considerably cheaper.

microRNA microarrays are commonly used in large-scale miRNA studies and several companies offer commercial arrays for miRNA expression analysis. miRNA arrays are rather new in the field of microarrays, and it has not been thoroughly established how to analyze the data. Because of the small size of the miRNome compared to the genome, one challenge is the normalization of miRNA microarray data (125, 206). Therefore, it is essential to validate miRNA microarray data with other techniques available.

1.6.2 Real-time PCR

Real-time PCR (quantitative real-time PCR/qPCR) is the most sensitive method to study the relative level of a specific RNA between two samples. Total RNA extracted from cells or tissues of interest is reversely transcribed to cDNA and then amplified using forward and reverse primers and analyzed in real-time. The data is measured and presented while the run is ongoing, thus the name ‘real-time’.

There are several different chemistries available for real-time PCR, the two most common being TaqMan and SYBR green. Both these chemistries generate fluorescence which will be relative to the amount of PCR product produced. TaqMan utilizes a sequence specific probe as well as primers specific for the gene of interest. The probe is an oligonucleotide containing a 5' fluorescent dye and a 3' quenching dye, designed to hybridize to an internal region of a PCR product. As long as the dyes are in close

proximity there will be no fluorescence. As the PCR product extends to the probe, it will be cleaved off by Taq polymerase and the dyes will separate resulting in fluorescence (127). SYBR green, on the other hand, binds to all double stranded DNA and will give a stronger signal as more DNA is amplified for each cycle of the PCR run. SYBR green primers therefore need to be carefully designed for optimal selectivity, and checked with melt curve analysis to ensure a pure PCR product, not containing unspecific fragments or primer dimerization. The advantages and disadvantages of the two techniques are that TaqMan is more specific but more expensive, while SYBR green is more cost effective but requires additional controls to ensure gene specificity.

Advantages of real-time PCR compared to microarrays are the sensitivity, specificity, and simplicity. However, some important considerations for real-time PCR are the need for a good reference gene and well designed, gene specific primers. This, together with the higher cost for primers and reagents for a large number of genes, limits the use of this method to studies exploring changes in a small set of genes such as confirmation of microarray data, rather than for a screening study of genome-wide changes. However, this limitation has somewhat been bypassed through the development of large scale real-time PCR plates. Real-time PCR plates already containing optimized gene specific primers covering either parts of the genome, a specific pathway or genes involved in a specific disease, are commercially available. The TaqMan low density arrays (TLDA) microRNA cards from Applied biosystems, covering most known miRNAs, are an example of large-scale analysis of the miRNome. One limitation with large-scale real-time PCR analysis is the decreased accuracy and specificity for low copy number genes, resulting in higher ΔC_t variances between intra-plate assays compared to high copy number genes (166).

1.6.3 RNA sequencing

Both microarrays and real-time PCR are widely used in analyses of gene expression due to their simplicity and low price. However, a growing field for genome-wide analyses is RNA sequencing (RNAseq). RNAseq has both advantages and disadvantages where one of the biggest advantages is that while microarrays only can detect transcripts present on the array, RNAseq can detect known and unknown genes as well as different splice variants and mutations (168). However, the cost for RNAseq is much higher than a microarray experiment, and might lead to a reduction of biological replicates being analyzed. In addition, since microarrays have been around for almost two decades, strategies for analyzing the data with minimal biases have been optimized, while it for RNAseq still is under development. Another major drawback is the magnitude of data generated from an RNAseq run. RNAseq data analysis requires knowledge in bioinformatics and data programming. It will take some time to develop tools for easy analysis of the data, but the more research that is done with this technique, the simpler and cheaper it will be (168). Despite the present drawbacks for RNAseq, this is a growing field that will become more common in the future.

2 AIMS OF THESIS

The general aim of this thesis was to increase the overall knowledge about the functions of ER α and ER β by studying their effects on gene transcription, with focus on their implications in breast- and colon cancer cells. Specifically, our objectives were:

- I. To investigate the differences and similarities in transcriptional regulation by the two estrogen receptors in T47D breast cancer cells (Paper I).
- II. To dissect the ER α -driven regulatory networks, through analyzing miRNA expression following estrogen stimulation of ER α in T47D breast cancer cells (Paper II).
- III. To study the role of re-introduction of ER β in the human colon cancer cell line SW480, with focus on cell cycle regulation and impact in xenograft tumors (Paper III).
- IV. To gain a better understanding of ER β transcriptional regulation in three colon cancer cell lines through transcriptome analyses and bioinformatics (Paper IV).
- V. To explore ER β -driven changes of the miRNA pool and correlating mRNA regulations in SW80 colon cancer cells (Paper V).

3 METHODOLOGICAL CONSIDERATIONS

In any experimental system, significant considerations need to be taken into account when deciding on the methodological layout as well as for data interpretation. Considerations and limitations of some of the major methods and systems used in the studies in this thesis are described below. Other considerations are discussed throughout each study.

3.1 CELL LINES

The cell lines used in this thesis were immortalized from different breast and colon cancer tumors, and purchased from American Type Culture Collection (ATCC). Cell lines are a useful tool for *in vitro* models in cancer research since they are easy to grow and can be cultured infinitely. However, due to their continuous culturing they are prone to undergo genotypic changes thereby creating different subpopulations. Two different labs working with the same cell line might get different results depending on culture conditions and passage number of the cells.

In addition, cancer is a complex disease with diverse genetic backgrounds and many separate, activated signaling pathways leads to the progression of the disease. Therefore, an immortalized cell line cannot be seen as the perfect model system for that particular type of cancer. An alternative approach is to generate primary cultures directly from tumors. However, since primary cultures grow slowly and have a definite lifespan of just a few passages, the use of primary cultures is limiting when large amounts of cells are needed.

Since ER β expression decreases during breast and colon cancer progression, no cancer cell line exist that express significant amounts of endogenous ER β . The lack of immortalized cell lines expressing ER β is one of the challenges when studying its transcriptional regulation. We therefore used systems where we introduced ER β into the cells, thereby restoring the lost endogenous expression of ER β . These methods will most likely not fully restore the physiological situation of the lost ER β , but serves as an excellent tool for *in vitro* mechanistic studies and as guidance for future *in vivo* studies.

The two breast cancer cell lines used, MCF-7 and T47D, both express significant levels of endogenous ER α , and in our study express ER β under the control of an inducible tet-off system. The three colon cancer cell lines used, SW480, HT29 and HCT-116 have low or no expression of endogenous ER α or ER β , and were made to express ER β through lentiviral transduction.

3.2 BIOINFORMATICS

Bioinformatics is a powerful *in silico* tool to analyze vast amounts of data. In this thesis, bioinformatics have been employed for microarray analyses, for gene ontology overrepresentation analyses, for genome-wide comparisons of ChIP with our

microarray data, for identifying predicted miRNA mRNA targets, for incorporation of literature searches and for building regulatory networks and pathways. However, as with any other method there are some limitations to take into consideration. There are many databases freely available with large amounts of data from different *in vivo* and *in vitro* experiments. However, tissue and cell specific effects are often not accounted for, and data that is true in one cell line might not be true in another cell line, even if they both come from the same type of cancer. In addition, chromatin binding site studies for a particular transcription factor might be similar in different cell lines, but *will* differ depending on presence of e.g. cofactors and packing of the chromatin. miRNA target predictions are algorithms calculating the most statistical probable target. All the different miRNA target predicting sites have different algorithms, thereby resulting in different responses despite the same in-data.

Bioinformatics can be used to find new angles and insights on obtained *in vitro/in vivo* results, and build new experiments based on that.

4 RESULTS AND DISCUSSION

4.1 PAPER I: A GENOME-WIDE STUDY OF THE REPRESSIVE EFFECTS OF ESTROGEN RECEPTOR BETA ON ESTROGEN RECEPTOR ALPHA SIGNALING IN BREAST CANCER CELLS

ER α mediates a proliferative effect in estrogen responsive breast cancers, and therapies targeting ER α are used in the clinic. It has been suggested and demonstrated *in vitro* that ER β opposes this effect of ER α . Most patients that initially respond to ER α targeting therapies develop resistance over time. New therapies are needed, and ER β is a potential target. However, the clinical significance of ER β in diagnostics and treatment has not been established. Thus, there is a need for a better understanding on the underlying mechanism of the supposedly antiproliferative effect of ER β . In Paper I, we performed a genome-wide analysis of ER α and ER α /ER β transcriptional activity, with the aim to elucidate the different regulatory networks of the two receptors (251).

We used T47D cells and expressed ER β through a tet-off system. ER β was expressed for 24 hours prior 24 hours treatment of ICI or E2 in control cells and ER β -expressing cells. Changes in gene expression were studied using microarray and real-time PCR. Our analysis identified 1434 ER α regulated transcripts, out of which 998 genes were inhibited by ER β expression. In addition, we found a large proportion of genes regulated by ER α alone or by ER β . 80% of the 50 strongest ER α regulated genes were induced by E2, while the corresponding number for ER β was 26%. This illustrates that ER α predominantly had an upregulatory effect while ER β negatively modulated a majority of its targets. This pattern was also seen in the genes commonly regulated by the two ERs, where 76% were upregulated by ER α and attenuated by ER β . In addition to genes regulated both by ER α and ER β , we also detected altered expression of genes in ER β -expressing cells that were not affected by ER α , revealing an ER β specific regulation. After removing boarder-line ER α regulated transcript, we had a list with 49 transcripts regulated by ER β alone, out of which 71% were upregulated.

Gene ontology analysis of all ER α regulated genes showed that the most overrepresented category for downregulated genes was related to the 'cell cycle', and for upregulated genes to 'cell adhesion', 'oncogenesis' and 'apoptosis'. A corresponding analysis for all ER β regulated genes showed an enrichment of downregulated genes within 'cell proliferation' and an enrichment of upregulated genes within 'negative regulation of cell growth' and 'apoptosis'. Further, a parallel analysis of genes regulated both by ER α and ER β showed an enrichment of genes within the 'cell cycle'. The fact that gene ontology analyses for both ERs showed an enrichment of genes within categories such as cell cycle and cell growth, further supported ER α 's proliferative effect and ER β 's antiproliferative effect. In addition, we could confirm this antiproliferative effect with a proliferation assay in which the estrogen stimulated ER α -induced proliferation in T47D cells was completely abolished when ER β was expressed.

Many of the ER α -regulated genes opposed by ER β , such as several cyclins (CCNA2, CCNB1, CCNB2, CCND1 and CNF), pS2, BCL2, GREB1, MYC and TP53INP1, are involved in proliferation and cell-cycle regulation. In addition, ER β alone regulated several antiproliferative genes, such as QSCN6, NDRG3, SEPT9, KCTD11 and STK3. IL-20 was one of the most strongly induced ER α -regulated genes that was opposed by ER β . IL-20 stimulates epithelial cell proliferation, antiapoptotic response, can enhance wound-healing activities and is involved in skin inflammations (192, 209). Deregulated IL-20 expression may lead to uncontrolled tissue repair processes resulting in diseases (192). Thus, the opposing effect by ER β on the proinflammatory cytokine IL-20 as well as the regulation of several genes involved in proliferation and cell-cycle regulation may therefore contribute to the antiproliferative properties of ER β .

In an effort to elucidate the mechanism behind ER β 's opposing effect on ER α signaling, we transfected T47D cells with an ER β protein with a mutated DNA-binding domain. We saw that the opposing action of ER β on some of the ER α regulated genes was in fact accentuated when ER β had a mutated DNA binding domain and was unable to bind to the DNA. One possibility is that ER β heterodimerizes with ER α resulting in reduced binding efficiency to the ERE compared to an ER α homodimer, thus explaining ER β 's opposing effect and lack of full inhibition of the ER α -driven transcription. However, when the mutated ER β heterodimerizes with ER α , the ability to bind to ERE is fully abolished.

Comparison of our T47D microarray data with previously published studies on ER α transcriptional regulation in MCF7 cells showed many common regulations (89). These were found despite the use of different cell lines, different treatments (ICI or vehicle) and different technology platforms. In addition to already published data, this study also contributed with a large number of novel ER α regulated genes, possibly cell specific. Few studies of ER β transcriptional regulation had been published at the time. Comparison with the one published study in, MCF7 breast cancer cells, found many discrepancies (45). This may be contributed to different cell lines, different mode of ER β delivery (tet-off construct versus transient adenoviral gene delivery) and the use of different microarray platforms. Nonetheless, both studies could show the same gene groups, such as cell-proliferation and ion homeostasis, being affected by introduction of ER β in ER α -expressing cells.

In conclusion, we could show that ER β opposed the effect of ER α -driven upregulation of genes involved in cell proliferation and cell cycle in T47D breast cancer cells, which we also functionally confirmed with a proliferation assay. Further, we demonstrated that ER β not only opposes ER α transcription, but also induced transcriptional changes independently of ER α .

4.2 PAPER II: ESTRADIOL-ACTIVATED ESTROGEN RECEPTOR α DOES NOT REGULATE MATURE MICRORNAS IN T47D BREAST CANCER CELLS

microRNAs (miRNAs) have been shown to be involved in several different cellular processes and is often deregulated in cancers, including breast cancer. Both mRNA and

miRNA are transcribed from the DNA by RNA polymerase II. Since estrogen can give rise to transcriptional regulation of protein coding genes in breast cancer (by RNA polymerase II), it is possible that miRNAs are transcribed by the same mechanism. Identification of miRNAs associated with disrupted estrogen signaling could therefore contribute to an enhanced understanding of the molecular processes underlying development of estrogen related breast cancer. Some studies have investigated ER α regulation of miRNAs in MCF7 breast cancer cells, but have presented conflicting results. In Paper II, we performed a thorough analysis of miRNAs regulated following 24 hours estrogen stimulation of T47D cells endogenously expressing ER α (137). We utilized several different techniques to identify changes in the miRNA pool. For the full miRNome analysis, we used both dual-color microarrays and TaqMan Low Density Arrays. Real-time PCR confirmations were then extensively performed with both SYBR Green and TaqMan chemistry.

First, we confirmed that 24 hours estrogen stimulation of T47D cells induced expected transcription of protein coding genes. pS2, KCNK5 and SPINK4 have all previously been shown to be regulated by ER α (251) and were therefore used as positive controls of gene transcription. We could confirm that 24 hours of estrogen treatment of T47D gave rise to a strong and significant transcriptional response on these genes. Further, treatment with ICI showed a decreased response on these genes. Thus, we could show that ER α induced transcription of mRNA in these cells. Our miRNA microarray revealed no significantly changed miRNAs after estrogen treatment whereas TLDA analysis detected 57 regulated miRNAs. However, these TLDA detected regulations could not be confirmed with real-time PCR (neither SYBR Green nor TaqMan).

Since the combined analysis with microarray, TLDA and real-time PCR did not show any significantly changed miRNAs, a literature study was performed to identify miRNAs previously indicated to be changed by ER α . Real-time PCR could not confirm an ER α -driven regulation on these miRNAs in T47D cells. Given that most published studies have been performed in MCF-7 cells, we also analyzed these miRNAs with real-time PCR in these cells. Despite the use of identical cell line, we could not show a direct regulation by ER α on these miRNAs. To further explore if estrogen exposure time would affect miRNA regulations, we performed times series with estrogen treatment in T47D cells. These studies did not show any change on miRNA regulation. This conflicts with previously published articles, highlighting the technical challenges when it comes to miRNA expression profiling.

In conclusion, even though there was a strongly induced estrogen-activated ER α -mediated gene transcription at 24 hours in T47D cells, no mature miRNAs were found to be significantly regulated.

4.3 PAPER III: TUMOR REPRESSIVE FUNCTIONS OF ESTROGEN RECEPTOR β IN SW480 COLON CANCER CELLS

The two estrogen receptors are sometimes co-expressed, often in varying ratios in different cell types. The development of estrogen dependent cancer can be facilitated

by changes in ratio of the two receptors. Both ER α and ER β are expressed in the normal breast epithelia but as breast cancer progresses, the ratio between them changes, often by a decrease of ER β and an increase of ER α . It is therefore of interest to study transcriptional effects by both receptors expressed in breast cancer cells. However, in the normal colonic epithelium where levels of ER α are significantly lower or not expressed at all, ER β is the major estrogen receptor, and is often decreased or lost as colon cancer progresses. Hormone replacement therapy has been shown to reduce the risk for colon cancer, implying a protective role for estrogen in development of colon cancer. This points towards a potential tumor suppressor function of ER β in colon cancer. For that reason, it is of interest to study the effect of ER β alone in colon cancer cells. The aim of Paper III was to study the molecular effect of reintroduction of ER β in SW480 colon cancer cells and especially its implication on cell cycle regulations (112).

Following lentiviral transduction with an ER β -expressing construct in SW480 colon cancer cells, proliferation was significantly decreased compared to control cells. FACS analysis confirmed this, showing that 75% of the ER β -expressing cells were halted in G₁ phase while the equivalent number for the control cells was 52%. Cells transfected with an ER β DBD-mutant did not show this antiproliferative effect, and resulted in a nearly complete removal of the ER β -driven G₁ arrest (58%). This demonstrates that the ER β -driven antiproliferative effect in SW480 colon cancer cells was ERE-dependent.

To corroborate the *in vitro* proliferation results *in vivo*, we performed a xenograft study with SW480 ER β and control cells in immunodeficient mice. This study showed a reduction in tumor weight by 65% in the ER β -expressing cells. Apoptosis was not affected by ER β , as demonstrated with a TUNEL assay, which made us conclude that a changed apoptosis potential was not the cause of the smaller size of the ER β -expressing xenografts. However, ER β -expressing tumors exhibited a significant reduction of Ki67 positive cells compared to control xenografts, confirming that ER β mediated a reduced proliferation, resulting in the smaller sized xenograft tumors.

Western blots revealed ligand-independent effects by ER β . This may be caused by the overexpression of ER β , but alternative causes will be further discussed in a section of Paper IV.

Stimulation of cells with growth factors will result in upregulation of cell-cycle and cell proliferation genes. One early response gene is MYC, and this gene was strongly downregulated by ER β . MYC has a central role in colon cancer growth (238) where it is an activator of many G₁-S phase genes. The ER β -driven downregulation of MYC is thus an important event contributing to the antiproliferative function of ER β in SW480 cells. Other key regulators of cell cycle are cyclin-dependent kinases (CDKs), where CDK2, 4, 5 and 6 are involved in G₁ phase progression. Western blot showed that the MYC target gene CDC25A was repressed in ER β -expressing cells. CDC25A activates CDK2 and CDK4; thus, low expression of this protein results in high levels of the inactive form of CDK2/CDK4 thereby halting the cells in G₁ phase. Further, ER β -induced downregulation of the p45 protein, a regulator of p27 degradation, resulted in a strong induction of the p27 protein. ER β also upregulated p14^{ARF}, a tumor suppressor involved in cell-cycle arrest. p14^{ARF} in turn downregulates MDM2, resulting in a stabilization of the p53 protein that, consequently, was strongly induced in ER β -

expressing cells. p53 promotes p21 expression, which also was upregulated. The two upregulated proteins p27 and p21 function as CDK2 inhibitors. ER β expression did not induce a change on p15 and p16. Cyclin D1 was upregulated by ER β , while cyclin E and A, both important in late phase of G₁, were not. Figure 6 illustrates CDKs and regulators of cell cycle progression, as well as the effect ER β expression had on respective protein level, indicating that the antiproliferative effect mediated by ER β is primarily through halting the cells in G₁ through the changed expression of several CDK regulators.

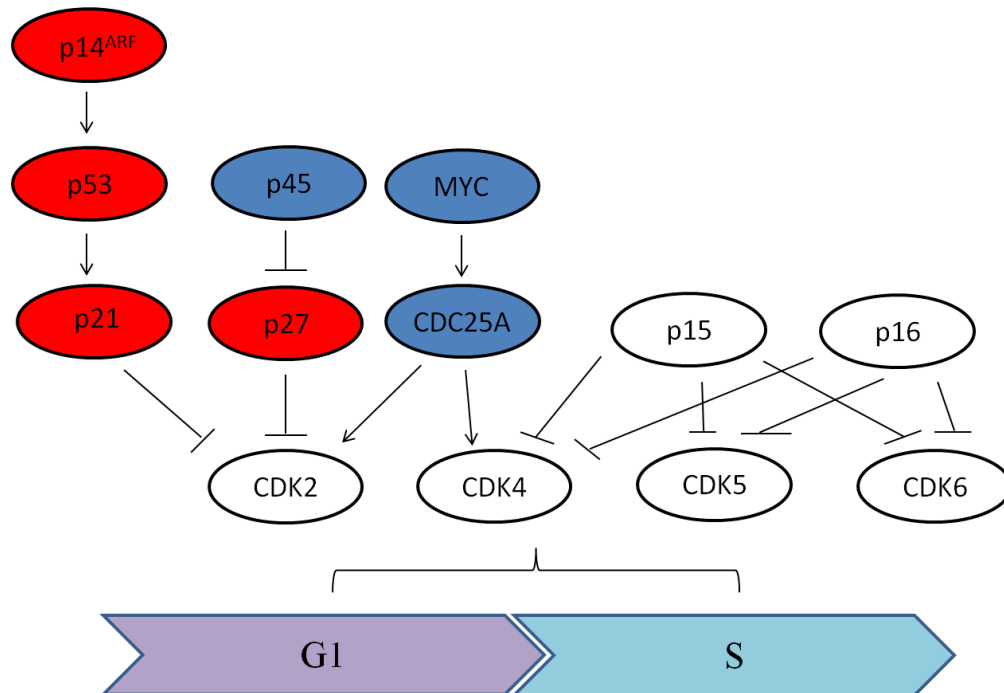


Figure 6. The cyclin-dependent kinases CDK2/4/5/6 are all important in regulation of cell cycle and the G1/S progression. Several CDK regulators were changed following ER β expression (genes in red were upregulated, and genes in blue were downregulated), suggesting an explanation for the G1 phase arrest and following decrease in proliferation in SW480 colon cancer cells.

Taken together, ER β demonstrated an antiproliferative role in SW480 colon cancer cells *in vitro* as well as in xenografts and affected proliferation partly through transcriptional regulation of genes involved in cell cycle regulation such as MYC, p14^{ARF}, p53, p21, p45, p27 and CDC25A. Further, we saw that ER β had significant ligand-independent effects.

4.4 PAPER IV: ESTROGEN RECEPTOR β INDUCES ANTI-INFLAMMATORY AND ANTITUMORIGENIC NETWORKS IN COLON CANCER CELLS

Introduction of ER β in both breast and colon cancer cells results in reduced proliferation and changes in key cell cycle proteins (112, 230, 251). The genome-wide study performed in T47D cells (Paper I) dissected ER β transcriptional effects in combination with endogenously expressed ER α . ER β genome-wide regulation on its

own is, however, not known. To further elucidate the mechanism behind ER β 's antiproliferative effect in colon cancer cells, and regulation by ER β homodimers alone, we performed a genome-wide analysis on three colon cancer cell lines with induced expression of ER β (Paper IV) (70).

Stable ER β expression resulted in large genome-wide transcriptional changes in all three cell lines. Although the impact of ER β was significant, few regulated transcripts were found in common between the different cell lines. Just like there were differences in ER β regulated genes in our T47D study compared to published ER β regulation in MCF-7 cells, we here demonstrated that ER β gene regulation is cell specific also in different colon cancer cells. In an effort to further understand ER β regulations, we analyzed enriched gene ontology groups in the three cell lines. Even though different genes were regulated, the GO analysis showed an enrichment of genes within protein binding, apoptosis, regulation of cell cycle, cell differentiation and kinase activity in all three cell lines. ER β therefore seems to regulate similar biological events, but uses different genes in the process. Cell specific activity could be caused by already activated pathways, mutations, presence of coregulators and cell specific genes in that particular cell. Some known differences in these three cell lines are mutations in p53 and varying levels of RAS, PROX1, MYC and DNA repair genes.

Once established that there were large variations between the different cell lines, we focused our studies on SW480 colon cancer cells. Enriched sub-network analysis revealed regulation of many downstream targets of IL-6. Real-time PCR could confirm a strong ER β -driven downregulation on IL-6. In addition, we also found reduction of mRNA levels of downstream targets (SP-1, VEGF, JUN1) in the MAPK/ERK pathway in ER β -expressing cells in the two other colon cancer cell lines HT29 and HCT-116. Previously we have revealed that ER β opposed the ER α upregulatory effect on IL-20 in breast cancer cells, and here we have shown that ER β downregulates IL-6 and many target genes in the MAPK/ERK pathway. This suggests a role for ER β in inflammation. A randomized clinical trial with hormone therapy as treatment for rheumatoid arthritis in post-menopausal women showed suppressed signs of inflammation (60), supporting the role for estrogen in treatment of inflammation. A key component in inflammatory signaling is NF κ B. The ERs are known to inhibit NF κ B inflammation response through several different pathways such as competing for the transcription coactivator CREB binding protein (CBP) (110), prevention of NF κ B DNA binding (106) or as a positive transcriptional cross-talk between NF κ B and ER (90). Most of these studies have however been performed in ER α positive breast cancer cells, and the mechanism behind the potential inhibitory role of ER β on NF κ B inflammation response in colon cells is not well studied. There is a connection between inflammation and tumorigenesis, where chronic intestinal inflammation proceeds tumor development. An ER β -mediated regulation of the inflammatory pathways could, in normal colonic epithelia *in vivo*, significantly contribute to the suggested colon cancer protective effect of estrogen and should be examined more thorough.

PROX1 is a transcription factor and nuclear receptor coregulator. It is often overexpressed in colon adenomas, in which high PROX1 expression is associated with poor tumor differentiation (199, 224). We found that re-expression of ER β in SW480 colon cancer cells led to a downregulation of PROX1 both at mRNA and protein level.

To our knowledge, this is the first time it has been shown that ER β expression can affect PROX1 levels. It is plausible that the ER β -driven downregulation of PROX1 is responsible for some of ER β 's antitumorigenic properties. Comparison of ER β -regulated genes to a PROX1 silencing microarray study (199) revealed many commonly changed genes, suggesting that regulation of these genes was a direct consequence of the ER β -driven downregulation of PROX1. Further, PROX1 chromatin binding sites (as published (46)) were enriched in the proximity of ER β regulated genes. Interestingly, several of these transcripts had both a PROX1 and an ER β binding site. With PROX1-ChIP we could confirm CITED2 as a gene regulated by both PROX1 and ER β , with a PROX1 binding site, in SW480 cells, less than 50 kb from TSS. It is therefore possible that PROX1 and ER β compete and/or co-regulate each other to influence the regulation of common target genes.

An ideal model to study the role of ER β in colon cancer would be a colon cancer cell line endogenously expressing ER β . However, to our knowledge no such cell line exists. We therefore need to rely on a model system where we express ER β . The model we used, with stable expression of ER β in mixed cell populations, made it impossible to distinguish between directly regulated transcript and secondary events. As a part of the analysis we utilized data from an ER β binding study in MCF-7 cells with the assumption that ER β binding sites in MCF-7 also exist in SW480, and compared it to our genome-wide data. Based on the MCF-7 dataset, 11% of the genome had an ER β binding site. We found an enrichment of genes with an ER β binding site in our ER β regulated genes (17%). The regulated genes that possessed an ER β binding site are potentially directly regulated by ER β .

Further, no ligand-dependent ER β regulatory effects were detected. Several studies have published ligand-independent gene regulations by both of the ERs. Mechanism behind this is not fully understood, but includes phosphorylation of the AF-1 domain via the MAPK pathway and ligand-independent association with the steroid receptor coactivators SRA, SRC and CBP to the AF-1 domain (65, 236, 237). We suggest that the observed ER β ligand-independent regulation is caused by high MAPK phosphorylation activity. A recent study showed that estrogen stimulated ER β had a protective effect in noncancerous colonocytes (243). We hypothesize that ER β has a ligand-dependent protective role in normal colon epithelial cells, but that this ligand-dependence for some unknown reason is lost upon re-expression in colon cancer cell lines.

In conclusion we have shown that reintroduction of ER β in colon cancer cells resulted in large and cell specific transcriptional changes. These regulations reduced proliferation and tumorigenic potential and included, for instance, genes in the MAPK/ERK signaling pathway. We could also conclude that the colon carcinogenic activity of PROX1 in SW480 cells was affected by ER β in dual ways; through down-regulation of PROX1 itself, and through co-regulation of the target genes.

4.5 PAPER V: ESTROGEN RECEPTOR BETA EXPRESSION INDUCES CHANGES IN THE MIRNA POOL IN HUMAN COLON CANCER CELLS

No studies so far have looked at a possible ER β regulation of miRNAs. We speculate that some of ER β 's antiproliferative effect and some of the transcriptional mRNA changes we detected after ER β re-expression may be attributed to miRNA regulations. In Paper V, we performed a large-scale miRNA analysis in SW480 colon cancer cells that stably expressed ER β , and correlated the changes to the transcriptome analysis previously performed (70).

We could show that ER β expression induced significant changes in the miRNA pool in SW480 cells, and defined 25 downregulated miRNAs and 3 upregulated miRNAs using miRNA real-time PCR confirmations. To investigate the impact of the changed miRNA pool, we used bioinformatics to identify predicted target genes and related these to the regulated mRNAs (70). We found an extensive overlap of predicted miRNAs targets and ER β regulated mRNAs. We believe that this finding can serve as a guide for future studies exploring the complexity of ER β transcriptional regulation.

As a first step to examine direct ER β regulatory effects on the changed miRNAs, we investigated whether or not there were ER β binding sites nearby the chromosomal location of the miRNAs. We found several miRNAs with proximal and distal binding sites, and 66% of the pre-miRNAs had an ER β binding site within 100kb. This indicated a possible direct ER β regulation for many of the regulated miRNAs. Other miRNAs may not be primary targets of ER β , but rather secondary events of ER β -regulated transcription factors. MYC is downregulated by ER β via a chromatin binding site less than 70kb from its promoter (100, 262). MYC is a key regulator of miRNAs (38, 140, 186) and miR-200a, miR-200b, miR-9-3, miR-106a, miR-221, miR-135a-1 and the miR-17-92 cluster have all been shown to be regulated by MYC in other cell lines. Thus, these twelve miRNAs may be changed as a consequence of the ER β -driven downregulation of MYC in SW480 cells.

miR-200a and miR-200b were strongly downregulated in ER β -expressing cells. The expression of these miRNAs is decreased in some cancers (68). They have both, in other cell lines, been shown to target ZEB1 and thereby lead to the upregulation of CDH1 (E-cadherin). Thus, the miR-200 family is believed to be involved in tumor suppression through the inhibition of the epithelial-mesenchymal transition (EMT) therefore inhibit formation of metastases. Using miR-200a and miR-200b mimics, we confirmed ZEB1 to be a target gene, resulting in the subsequent upregulation of E-cadherin. This regulation does not explain the protective role of ER β , and the impact of miR-200 downregulation in colon cancer needs to be further explored. However, it has been demonstrated that miR-200 expression also can promote metastatic properties of some cancer cells, suggesting that changed expression of the miR-200 family will have different impact on disease outcome depending on cellular context (68).

Another interesting finding was the strong downregulation of the miR-17-92 cluster, also known as Oncomir-1, consisting of miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92. This cluster is known to act as an oncogene (116) and has been shown to be involved in enhanced cell proliferation and suppression of apoptosis in human B-

cell lymphomas and in colon cancer cells (66, 116). The strong downregulation of this cluster may contribute to the complex pathway in which ER β generates the antiproliferative effect, as demonstrated in the xenograft study (112). We identified and confirmed that the ER β regulated genes CLU and NCOA3 (SRC-3) both were induced when miR-17 was silenced. NCOA3 is reported to affect the expression of other transcription factors and regulators such as NCOA1 (SRC-1), AHR, PPARG, CEBPD and MYB. These genes were all changed in ER β -expressing cells, pointing towards an ER β -driven regulation of these genes partially through the downregulation of miR-17 and the subsequent upregulation of NCOA3. NCOA3 is a nuclear receptor coactivator, affecting the transcriptional activity of both ER α and ER β (156, 254). NCOA3 has been associated with a more locally advanced disease in CRC, and elevated levels of the protein have been reported in many different cancers (258) (99). However, elevated levels of NCOA3 have also been found to be associated with a better overall survival in CRC (99). Therefore, the role of NCOA3 as an ER β coregulator and the upregulatory effect of ER β regulated miRNAs on NCOA3 in colon cancer needs to be explored further.

We also found that treatment with cisplatin, a DNA damaging agent, led to a higher cell death in miR-17 silenced cells compared to wild type cells. This finding indicates an interesting role for co-treatment with miR-17 inhibitor and chemotherapy in colon cancer patients resistant to chemotherapy.

In conclusion, we have shown that stable re-expression of ER β in SW480 colon cancer cells resulted in a large change of the miRNA pool. To our knowledge, this is the first report of ER β affecting miRNAs. We further detected changed mRNA levels of many miRNA predicted target genes, illustrating the impact of the ER β regulated miRNA pool on mRNA levels.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

ER α was discovered more than 50 years ago and major efforts have been put into describing the molecular network by which it works. The regulatory networks of ER α are therefore relatively well studied, and ER α is used as a therapeutic target in breast cancer. ER β was discovered 15 years ago, and the molecular mechanisms behind ER β 's effect are still far from clear. Normal breast epithelia expresses both ER α and ER β , but the ratio between them changes during cancer progression. A standard procedure in breast cancer diagnostics consists of identifying those patients expressing ER α , thereby more likely to respond to tamoxifen treatment. At this time, ER β is not used in diagnostics, and the clinical significance of ER β in breast cancer has not been established. Clinical trials with progestin-estrogen HRT indicate a protective role against colorectal carcinoma. As ER β is the predominant ER in the colonic epithelium and is lost during cancer progression, it is reasonable to assume that these protective effects are conveyed by ER β . This has further been supported *in vivo* studies where deletion of ER β in mice that spontaneously develops intestinal adenomas leads to an increase both in size and number of adenomas (94). Despite these facts, the role of ER β in colon cancer is not well explored.

Since ER α has been shown to be involved in the development and progression of breast cancer, and ER β has been indicated to mediate a protective effect in colon cancer, it is of importance to elucidate the molecular mechanism behind their tumorigenic and anti-tumorigenic properties, respectively. The aim of this thesis was to dissect the regulatory networks of ER α and ER β and thereby gain a better knowledge of the molecular and cellular mechanisms of estrogen signaling in breast and colon cancer. A better understanding of the regulatory networks by which ER α and ER β work, would not only aid in development of novel therapeutics based on downstream targets of the ER pathways but also help identify those patients that might benefit from treatment aiming at ER α and/or ER β .

When this study was initiated, it had been established that ER α was proliferative. It had been suggested that ER β was antiproliferative, but this notion had not yet been widely accepted. We could show that ER β often opposed the transcriptional effect of ER α at the transcription levels in T47D cells, especially for genes within proliferation and regulation of cell cycle. We also found a set of genes regulated by ER β alone, indicating that in spite of the high homology in their DBDs, there are differences in their transcriptional targets. The significance of ER β in breast cancer has been debated, but our data implies that ER β may play an important role in the treatment and diagnostics of breast cancer. Stimulation of ER β through ER β specific agonists is one future direction for the treatment of estrogen responsive breast cancers. However, it is not established to what extent ER β is expressed in breast cancer tumors due to the varying specificity and efficiency of commercially available ER β antibodies. Therefore, there is a need for development of new ER β antibodies with a better specificity. This would facilitate further exploration of ER β occurrence in breast cancer tumors and lead to a better knowledge if ER β is expressed at those levels that ER β targeting therapies would be beneficial as an alternative treatment method.

Most studies on ER β so far had been performed in cell lines expressing endogenously ER α in addition to the transduced ER β . Therefore, they could only detect how ER β behaved in combination with ER α . We demonstrated that ER β not only is antiproliferative when co-expressed with ER α in breast cancer, but also by itself in colon cancer cells. We further showed that this also was true *in vivo* with xenograft ER β -expressing tumors. Our studies substantially contribute to a better understanding of the mechanism behind ER β 's antiproliferative effect in colon cancer cells by the discovery that ER β halts the cells in G₁-phase through the strong regulatory inhibition on MYC and several other cell cycle genes.

ER α has been widely studied, and there are several accepted estrogen target genes. However, no transcriptional profile had been established and accepted for ER β . To further explore the impact of ER β regulation in colon cancer, we performed genome-wide analyses. This showed that ER β is capable of regulating a large set of genes independently of ER α , and that the set of ER β regulated genes is cell type and cell line specific. We therefore could not establish one specific ER β transcriptional profile in colon cancer cells. However, the different sets of ER β regulated genes in three different colon cancer cell lines were all involved in similar biological processes, such as regulation of cell cycle, kinase activity and apoptosis. We therefore suggest that ER β regulates similar biological processes in different cells, but uses different pathways and genes to reach them. The exact mechanism behind ER β 's regulatory network in colon cancer cells is still not clear, but some key factors such as regulation of cell cycle, MAPK signaling and PROX1 play important roles. The biological role of these target genes in the development and progression of colon cancer as well as correlation between them and ER β expression should be further explored in normal colon cells. In addition, for a full understanding of the ER β regulatory pathways, future studies should include a thorough analysis of ER β binding sites in colon cells, such as with RNAseq and ChIP-Seq, as well as proteomic studies and analysis of post-translational modifications, especially involving ER β regulation on kinases and subsequent effect on protein phosphorylations.

The finding of gene inhibition by miRNAs is rather new in the field of gene regulation. As the number of known miRNAs increases for each year, the understanding of their role in different biological pathways and processes is improved. miRNAs are involved in most biological processes and are often deregulated in both breast and colon cancer. One recent study showed that expression of the mir302/367 cluster efficiently could reprogram human and mouse fibroblasts to an induced pluripotent stem cell state (12). This demonstrates that changes in the miRNA pool of just a few miRNAs might be sufficient for a cell to switch into a different type of cell, or possibly turn cancerous. Identification of miRNAs that are associated with normal or disrupted estrogen signaling would be of great importance for prognosis and diagnosis. Previous studies on ER α regulation of miRNAs in breast cancer cells revealed conflicting results. We could show a strong estrogen activated ER α transcriptional response in T47D cells, but we did not detect any rapid changes of mature miRNA expression. However, we found that stable re-expression of ER β in colon cancer cells led to a significant change of the miRNA pool. Many of the changed miRNAs were indirectly regulated via MYC, but others had an ER β binding site within 100kb indicating a possible direct ER β regulation. This finding offers insight into the long term effect of ER β activation in

colon. In addition, we could show that genes regulated by ER β could be enhanced through ER β regulated miRNAs, illustrating how ER β can fine tune gene expression through the regulation of miRNAs. To our knowledge, this is the first study presenting miRNAs regulated by ER β . This opens up for an entirely new field of potential therapeutic targets of ER β signaling in colon cancer. Future mimic and inhibitor studies, *in vitro* and *in vivo*, with detected changed miRNAs are interesting approaches to further explore miRNAs as potential therapeutic targets in estrogen dependent colon cancers.

The findings in this thesis significantly contribute to elucidating the regulatory pathways of the two estrogen receptors. We now know, e.g., that ER β not only opposes the effect of ER α but also induces transcriptional response as a homodimer, that ER β is antiproliferative in both breast and colon cancer cells, partly through ER β -induced inhibition of PROX1, MYC and other G1-phase cell cycle genes, that ER β may fine-tune mRNA expression through the regulation of miRNAs and that ER β has anti-inflammatory properties. All this supports the notion that ER β is a potential target in breast and colon cancer prevention and/or inflammation proceeding CRC development. ER β expression might be lost in many cancers especially at later stages, thus not a possible target for novel therapeutics. One approach would be reintroduction of ER β in these tumors. However, this is currently not a practical or realistic solution. Instead, investigations should be done to explore if specific ER β agonists have treatment potential or if novel therapeutics could be aimed at targets downstream of ER β , such as those discovered in this thesis.

Only a few studies have explored the use of ER β specific ligands or phytoestrogens in animal studies, and just one small pilot study has explored the combinatory effect of two phytoestrogens on CRC in humans. Given that we have shown that ER β exhibits an antiproliferative effect we suggest that a specific ER β agonist might prove to be an interesting complement in future therapies against early stages of ER β -expressing breast and colon cancer tumors as well as in treatment of inflammation. In addition, ER β specific agonists may potentially be used as a preventive approach for persons with an increased risk of developing CRC, such as IBD patients. Undoubtedly, there is a need for development and characterization of ER β specific ligands to be used *in vitro* and in subsequent *in vivo* randomized clinical trials to fully understand and utilize the clinical significance of ER β in estrogen responsive cancer.

6 ACKNOWLEDGEMENTS

This thesis work was performed at the Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden (December 2007 - March 2009, July 2011 - December 2011) and at the Center for Nuclear Receptors and Cell Signaling, University of Houston, Houston, Texas, USA (March 2009 - June 2011). I would like to express my deepest appreciation to all past and present colleagues and friends who have helped me during these four years, thus making this thesis possible. I would especially like to thank:

My supervisor **Cecilia Williams** for accepting me as PhD student. Thank you for sharing your knowledge and your endless support. Thanks for your never ending optimism and all rewarding discussions we have had. Thanks for guiding me into becoming an independent scientist, for giving me the possibility to help set up your lab in Houston and finally thank you for letting me stay with your family my first few weeks in Texas.

Lars-Arne Haldosén, my co-supervisor, for your scientific advices and for sharing your knowledge. I am grateful for all your support and guidance.

My professor, **Jan-Åke Gustafsson**, for inspiration and scientific guidance. Thanks for the excellent research facilities you have provided. I am especially grateful for the opportunity to participate in the set-up of the new research facilities at CNRCS in Houston.

I would especially like to thank all collaborators that significantly have contributed to make this thesis possible: **Anders, Anne, Chunyan, Eylem, Fredrik, Johan, Karolina, Philip, Sebastian, Sharanya** and **Trang**.

To my fellow graduate students, colleagues and friends at Novum:

Agneta, for being a good group leader, and for your support the last six months. **Chunyan**, for all discussions regarding analysis of bioinformatics data. **Eckardt**, for sharing your scientific knowledge and for your humor. **Fredrik**, for nice lunch company and for quickly analyzing my samples with the Bioanalyzer just a few days before Christmas. **Gayathri**, for being the sweet person you are, and for all nice talks we had when we shared an office. **Hui**, for advice on ChIP experiments and for nice company in the lab. **José**, for a good and inspiring collaboration. **Karin**, for creating a good lab environment when I first came to Novum. **Kirsten**, for nice company in the office and the everyday conversations we've had. **Knut**, for fun company at the pubs, and for your scientific knowledge. **Lotta**, for all scientific discussions and advice on lab work, for being a great friend and for all fun adventures during our beer trips (I am looking forward to our beer-interrailing anniversary trip in 2058). **Malin**, for nice company during lunches. **Marika**, for letting me share the pre-PCR room (closet). **Milica**, for fun times in the lab, for all scientific advices, and for joining me in the hunt for a good Mojito in Београд. **Nina**, for great company during fika and dinners. **Patricia**, for your warm personality and nice conversations. **Patrick**, for entertaining company during lunches and fika. **Per**, for nice lunch company. **Qaraman**, for nice

company in the office. **Tassos**, for fun chats when we shared office, and for creating a better work environment by always making sure there is freshly made coffee in the mornings. **Tomas**, for fun talks in the office about everything but science, when we both really should be writing, and for taking the time to answer my never ending questions regarding the dissertation process. **Treska**, for being a nice office buddy.

To the administrative staff and especially to **Monica** and **Lena** for all their help while I was in Texas.

To all other members of AMO, KDW, BEA: none mentioned, none forgotten.

To past colleagues: **Anna**, for being a great scientist, for all rewarding scientific discussions and fun times outside of work. I still think we should make that GFP-beer. **Jingwen**, for being a great friend and always spreading sunshine around you. **Karolin**, for your friendship, fun times in the lab, entertaining dinners and our crazy beer trip. **Karolina**, for great company at work, for all fun dinners and for being a wonderful friend, always with an ear to lend. We really have to go out and “untz, untz” soon! **Lovisa**, for nice company in the lab and fun dinners with the receptor girls. **Marcela**, for fun times in the office, for your never ending energy and for the lovely necklace you made me. **Susanne**, for a good collaboration and interesting discussions. **Zoi**, for your bubbly personality, always with a smile to spare.

To all my colleagues and friends at University of Houston:

Anders, for sharing your knowledge, for teaching me cell culturing and for all the help with the cell lines. **Anna**, for always finding time to help me no matter how much work you had, and for being such an inspiration to young girls at GirlsRockCamp! **Anne**, for being great company in the lab and at fikas. **Armando**, for all your help in the lab and for always suggesting solutions to my problems. **Catherin**, for your cheerful personality and for liking my Pandan cake. **Chin-Yo**, for your great scientific knowledge, your honest opinions and your humor. **Christoforos**, for your friendship, scientific discussions and for sharing your view of the world :). **Efi**, for being a sweetheart and the most honest person I know. **Eylem**, for great scientific discussions and fun times outside of the lab. **Fotis**, for all fun times in and outside of the lab. **Gayani**, for creating a warm lab environment with your sweet personality. **Heather**, for your friendship and for letting me participate in your wonderful wedding. **Hyunjin**, for being a great friend, fun times in the lab and for showing me Korea house. **Itala**, for being the best IT-support in the world. **Lara**, for introducing me to the world of ants, and for taking me out hiking. **Lindsey**, for your fun personality and fun company during lunches, fikas and dinners. **Lucy**, for your knowledge and fun times outside of the lab. **Maria**, for sharing your knowledge and for being such an enthusiastic and nice person. **Nick**, for creating a nice work environment, for being a great friend, for all the laughter and fun times out clubbing. **Philip**, for “monitoring” the overall environment in the lab, for sending out angry e-brev, for magnificent scientific discussions, for being a great friend and for all fun times outside of the lab. **Prasenjit**, for great scientific discussions and help in the lab. **Sharanya**, for creating a nice lab environment with your kind personality. **Sabrina**, for nice lunch company and for your cheerful personality. **Stefan**, for fun and entertaining chats during lunch and fika. (Her Majesty Royal Highness Most-Awesome-Tex-Nameese) **Trang** for groovy scientific

discussions, for being my little helper when I needed more experiments done, for your friendship and for sharing the video of 1.0 singing “Helan går”.

To all fellow graduate students at the center, especially: **Alicia, Jinyu, Lakshmi, Ryan Ruixin, Kim, Shana, Caroline**. Thanks for all good scientific discussions you're your support. Y'all have made the lab a more fun place to work at! I wish you all the best.

To the administration, and all other lab members of the groups Gustafsson/Warner, Lin, Frigo, Chung, Zhang W and Zhang S: none mentioned, none forgotten.

To all my friends outside of the world of science:

Friends in Sweden: **Anna**, for your friendship and fun times at the shooting range. **Bambi**, for all fun techno cruises. **Bea**, for nice dinners together and great conversations about life. **Elias**, for being a great friend and listener. **Ewa**, for your friendship and inspirational personality. **Johan**, for your friendship and for always believing in me. **Jossan**, for always supporting me in everything I do, and for being my personal hairdresser. **Kia**, for being friends for over 20 years. **Maria**, for shared laughter and fun times together. **Micke**, for being a good friend and fun travel companion in Japan. **Sara**, for your friendship and support, and for all the laughter we have shared. **Smurf**, for always being there for me and for fun adventures during your visit in Houston. **Stella**, for taking me wherever I needed to go. (Manga-) **Tina**, for a long lasting friendship and for tasty chocolate making sessions. (Riktiga-) **Tina**, for always bragging about me to all your friends and for your bouncy and happy personality. (Big Brother-) **Victoria**, for fun wine nights, for always making me laugh and for being a great friend.

Friends in Texas: **Alexa** and the boys, for being the best roommates ever, for taking the time to explain the American mentality and for turning me into a crazy pug lady. **John, Melissa** and **LaKitia** for entertaining dinners and fun times at Oktoberfest. **Nicole** and **Ferrier** for fun company during those happy hours and dinners. **Patrick** – nowadays I'm wearing my beaver t-shirt when I'm baking Swedish carrot cake. **Edgar**, for showing me the nightlife of Houston. **Elaine**, for fun times during my last weeks in Houston. We'll meet again someday, somewhere. **Carlos**, for your entertaining travel updates.

My family:

My **mother, father, Oskar** and **Alexandra, Hasse, Per** and **Anna-Lena**; for believing in me and supporting me in everything I do.

Aleksi and **Marica Petkov**, for accepting me in your family and for showing me Croatia.

Alex, for patiently waiting for me during those 27 months with an ocean apart, for all exciting adventures and trips together and for always making me smile no matter how bad my day has been. Volim te dušo! <3

7 REFERENCES

1. Cancer Incidence in Sweden 2009, Socialstyrelsen (2010).
2. 1998. Design of the Women's Health Initiative clinical trial and observational study. The Women's Health Initiative Study Group. *Control Clin Trials* **19**:61-109.
3. <http://www.cancerfonden.se/sv/cancer/Cancersjukdomar/Brostcancer>, accessed 22/10/11
4. **Aagaard, M. M., R. Siersbaek, and S. Mandrup.** 2011. Molecular basis for gene-specific transactivation by nuclear receptors. *Biochim Biophys Acta* **1812**:824-35.
5. **Adams, B. D., H. Furneaux, and B. A. White.** 2007. The micro-ribonucleic acid (miRNA) miR-206 targets the human estrogen receptor-alpha (ERalpha) and represses ERalpha messenger RNA and protein expression in breast cancer cell lines. *Mol Endocrinol* **21**:1132-47.
6. **Adlercreutz, H.** 1998. Epidemiology of phytoestrogens. *Baillieres Clin Endocrinol Metab* **12**:605-23.
7. **Al-Nakhle, H., P. A. Burns, M. Cummings, A. M. Hanby, T. A. Hughes, S. Satheesha, A. M. Shaaban, L. Smith, and V. Speirs.** 2010. Estrogen receptor {beta}1 expression is regulated by miR-92 in breast cancer. *Cancer Res* **70**:4778-84.
8. **Alexander, D. D., and C. A. Cushing.** 2011. Red meat and colorectal cancer: a critical summary of prospective epidemiologic studies. *Obes Rev* **12**:e472-93.
9. **Alexander, D. D., C. A. Cushing, K. A. Lowe, B. Scurman, and M. A. Roberts.** 2009. Meta-analysis of animal fat or animal protein intake and colorectal cancer. *Am J Clin Nutr* **89**:1402-9.
10. **Ambros, V.** 2004. The functions of animal microRNAs. *Nature* **431**:350-5.
11. **Andreyev, H. J., A. R. Norman, D. Cunningham, J. R. Oates, and P. A. Clarke.** 1998. Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study. *J Natl Cancer Inst* **90**:675-84.
12. **Anokye-Danso, F., C. M. Trivedi, D. Jühr, M. Gupta, Z. Cui, Y. Tian, Y. Zhang, W. Yang, P. J. Gruber, J. A. Epstein, and E. E. Morrissey.** 2011. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell Stem Cell* **8**:376-88.
13. **Antoniou, A., P. D. Pharoah, S. Narod, H. A. Risch, J. E. Eyfjord, J. L. Hopper, N. Loman, H. Olsson, O. Johannsson, A. Borg, B. Pasini, P. Radice, S. Manoukian, D. M. Eccles, N. Tang, E. Olah, H. Anton-Culver, E. Warner, J. Lubinski, J. Gronwald, B. Gorski, H. Tulinius, S. Thorlacius, H. Eerola, H. Nevanlinna, K. Syrjäkoski, O. P. Kallioniemi, D. Thompson, C. Evans, J. Peto, F. Lalloo, D. G. Evans, and D. F. Easton.** 2003. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. *Am J Hum Genet* **72**:1117-30.
14. **Aoki, K., and M. M. Taketo.** 2007. Adenomatous polyposis coli (APC): a multi-functional tumor suppressor gene. *J Cell Sci* **120**:3327-35.
15. **Arpino, G., C. Gutierrez, H. Weiss, M. Rimawi, S. Massarweh, L. Bharwani, S. De Placido, C. K. Osborne, and R. Schiff.** 2007. Treatment of human epidermal growth factor receptor 2-overexpressing breast cancer xenografts with multiagent HER-targeted therapy. *J Natl Cancer Inst* **99**:694-705.
16. **Arpino, G., H. Weiss, A. V. Lee, R. Schiff, S. De Placido, C. K. Osborne, and R. M. Elledge.** 2005. Estrogen receptor-positive, progesterone receptor-negative breast cancer: association with growth factor receptor expression and tamoxifen resistance. *J Natl Cancer Inst* **97**:1254-61.
17. **Arpino, G., L. Wiechmann, C. K. Osborne, and R. Schiff.** 2008. Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. *Endocr Rev* **29**:217-33.
18. **Asselin-Labat, M. L., F. Vaillant, J. M. Sheridan, B. Pal, D. Wu, E. R. Simpson, H. Yasuda, G. K. Smyth, T. J. Martin, G. J. Lindeman, and J. E. Visvader.** 2010.

- Control of mammary stem cell function by steroid hormone signalling. *Nature* **465**:798-802.
19. **Atanaskova, N., V. G. Keshamouni, J. S. Krueger, J. A. Schwartz, F. Miller, and K. B. Reddy.** 2002. MAP kinase/estrogen receptor cross-talk enhances estrogen-mediated signaling and tumor growth but does not confer tamoxifen resistance. *Oncogene* **21**:4000-8.
 20. **Bain, D. L., A. F. Heneghan, K. D. Connaghan-Jones, and M. T. Miura.** 2007. Nuclear receptor structure: implications for function. *Annu Rev Physiol* **69**:201-20.
 21. **Bammler, T., R. P. Beyer, S. Bhattacharya, G. A. Boorman, A. Boyles, B. U. Bradford, R. E. Bumgarner, P. R. Bushel, K. Chaturvedi, D. Choi, M. L. Cunningham, S. Deng, H. K. Dressman, R. D. Fannin, F. M. Farin, J. H. Freedman, R. C. Fry, A. Harper, M. C. Humble, P. Hurban, T. J. Kavanagh, W. K. Kaufmann, K. F. Kerr, L. Jing, J. A. Lapidus, M. R. Lasarev, J. Li, Y. J. Li, E. K. Lobenhofer, X. Lu, R. L. Malek, S. Milton, S. R. Nagalla, P. O'Malley J, V. S. Palmer, P. Pattee, R. S. Paules, C. M. Perou, K. Phillips, L. X. Qin, Y. Qiu, S. D. Quigley, M. Rodland, I. Rusyn, L. D. Samson, D. A. Schwartz, Y. Shi, J. L. Shin, S. O. Sieber, S. Slifer, M. C. Speer, P. S. Spencer, D. I. Sproles, J. A. Swenberg, W. A. Suk, R. C. Sullivan, R. Tian, R. W. Tennant, S. A. Todd, C. J. Tucker, B. Van Houten, B. K. Weis, S. Xuan, and H. Zarbl.** 2005. Standardizing global gene expression analysis between laboratories and across platforms. *Nat Methods* **2**:351-6.
 22. **Bardou, V. J., G. Arpino, R. M. Elledge, C. K. Osborne, and G. M. Clark.** 2003. Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases. *J Clin Oncol* **21**:1973-9.
 23. **Barone, M., M. P. Scavo, S. Papagni, D. Piscitelli, R. Guido, M. Di Lena, M. C. Comelli, and A. Di Leo.** 2010. ERbeta expression in normal, adenomatous and carcinomatous tissues of patients with familial adenomatous polyposis. *Scand J Gastroenterol* **45**:1320-8.
 24. **Barone, M., S. Tanzi, K. Lofano, M. P. Scavo, M. Pricci, L. Demarinis, S. Papagni, R. Guido, E. Maiorano, G. Ingravallo, M. C. Comelli, A. Francavilla, and A. Di Leo.** 2010. Dietary-induced ERbeta upregulation counteracts intestinal neoplasia development in intact male ApcMin/+ mice. *Carcinogenesis* **31**:269-74.
 25. **Bartel, D. P.** 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**:281-97.
 26. **Bartel, D. P.** 2009. MicroRNAs: target recognition and regulatory functions. *Cell* **136**:215-33.
 27. **Becker, C., M. C. Fantini, C. Schramm, H. A. Lehr, S. Wirtz, A. Nikolaev, J. Burg, S. Strand, R. Kiesslich, S. Huber, H. Ito, N. Nishimoto, K. Yoshizaki, T. Kishimoto, P. R. Galle, M. Blessing, S. Rose-John, and M. F. Neurath.** 2004. TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity* **21**:491-501.
 28. **Bedeir, A., and A. M. Krasinskas.** 2011. Molecular diagnostics of colorectal cancer. *Arch Pathol Lab Med* **135**:578-87.
 29. **Berg, D. J., N. Davidson, R. Kuhn, W. Muller, S. Menon, G. Holland, L. Thompson-Snipes, M. W. Leach, and D. Rennick.** 1996. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest* **98**:1010-20.
 30. **Bernstein, E., S. Y. Kim, M. A. Carmell, E. P. Murchison, H. Alcorn, M. Z. Li, A. A. Mills, S. J. Elledge, K. V. Anderson, and G. J. Hannon.** 2003. Dicer is essential for mouse development. *Nat Genet* **35**:215-7.
 31. **Bhat-Nakshatri, P., G. Wang, N. R. Collins, M. J. Thomson, T. R. Geistlinger, J. S. Carroll, M. Brown, S. Hammond, E. F. Srouf, Y. Liu, and H. Nakshatri.** 2009. Estradiol-regulated microRNAs control estradiol response in breast cancer cells. *Nucleic Acids Res* **37**:4850-61.
 32. **Bjornstrom, L., and M. Sjoberg.** 2005. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol Endocrinol* **19**:833-42.

33. **Bledsoe, R. K., V. G. Montana, T. B. Stanley, C. J. Delves, C. J. Apolito, D. D. McKee, T. G. Consler, D. J. Parks, E. L. Stewart, T. M. Willson, M. H. Lambert, J. T. Moore, K. H. Pearce, and H. E. Xu.** 2002. Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. *Cell* **110**:93-105.
34. **Bocchinfuso, W. P., J. K. Lindzey, S. C. Hewitt, J. A. Clark, P. H. Myers, R. Cooper, and K. S. Korach.** 2000. Induction of mammary gland development in estrogen receptor-alpha knockout mice. *Endocrinology* **141**:2982-94.
35. **Bodmer, W. F., C. J. Bailey, J. Bodmer, H. J. Bussey, A. Ellis, P. Gorman, F. C. Lucibello, V. A. Murday, S. H. Rider, P. Scambler, and et al.** 1987. Localization of the gene for familial adenomatous polyposis on chromosome 5. *Nature* **328**:614-6.
36. **Brennecke, J., D. R. Hipfner, A. Stark, R. B. Russell, and S. M. Cohen.** 2003. bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in *Drosophila*. *Cell* **113**:25-36.
37. **Brozek, W., S. Kriwanek, E. Bonner, M. Peterlik, and H. S. Cross.** 2009. Mutual associations between malignancy, age, gender, and subsite incidence of colorectal cancer. *Anticancer Res* **29**:3721-6.
38. **Bui, T. V., and J. T. Mendell.** 2010. Myc: Maestro of MicroRNAs. *Genes Cancer* **1**:568-575.
39. **Bulger, M., and M. Groudine.** 1999. Looping versus linking: toward a model for long-distance gene activation. *Genes Dev* **13**:2465-77.
40. **Campbell-Thompson, M., I. J. Lynch, and B. Bhardwaj.** 2001. Expression of estrogen receptor (ER) subtypes and ERbeta isoforms in colon cancer. *Cancer Res* **61**:632-40.
41. **Campbell, R. A., P. Bhat-Nakshatri, N. M. Patel, D. Constantinidou, S. Ali, and H. Nakshatri.** 2001. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. *J Biol Chem* **276**:9817-24.
42. **Carroll, J. S., X. S. Liu, A. S. Brodsky, W. Li, C. A. Meyer, A. J. Szary, J. Eeckhoutte, W. Shao, E. V. Hestermann, T. R. Geistlinger, E. A. Fox, P. A. Silver, and M. Brown.** 2005. Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell* **122**:33-43.
43. **Carroll, J. S., C. A. Meyer, J. Song, W. Li, T. R. Geistlinger, J. Eeckhoutte, A. S. Brodsky, E. K. Keeton, K. C. Fertuck, G. F. Hall, Q. Wang, S. Bekiranov, V. Sementchenko, E. A. Fox, P. A. Silver, T. R. Gingeras, X. S. Liu, and M. Brown.** 2006. Genome-wide analysis of estrogen receptor binding sites. *Nat Genet* **38**:1289-97.
44. **Chambliss, K. L., I. S. Yuhanna, R. G. Anderson, M. E. Mendelsohn, and P. W. Shaul.** 2002. ERbeta has nongenomic action in caveolae. *Mol Endocrinol* **16**:938-46.
45. **Chang, E. C., J. Frasor, B. Komm, and B. S. Katzenellenbogen.** 2006. Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells. *Endocrinology* **147**:4831-4842.
46. **Charest-Marcotte, A., C. R. Dufour, B. J. Wilson, A. M. Tremblay, L. J. Eichner, D. H. Arlow, V. K. Mootha, and V. Giguere.** 2010. The homeobox protein Prox1 is a negative modulator of ERR{alpha}/PGC-1{alpha} bioenergetic functions. *Genes Dev* **24**:537-42.
47. **Charn, T. H., E. T. Liu, E. C. Chang, Y. K. Lee, J. A. Katzenellenbogen, and B. S. Katzenellenbogen.** 2010. Genome-wide dynamics of chromatin binding of estrogen receptors alpha and beta: mutual restriction and competitive site selection. *Mol Endocrinol* **24**:47-59.
48. **Cheng, G., Z. Weihua, M. Warner, and J. A. Gustafsson.** 2004. Estrogen receptors ER alpha and ER beta in proliferation in the rodent mammary gland. *Proc Natl Acad Sci U S A* **101**:3739-46.
49. **Chlebowski, R. T., J. Wactawski-Wende, C. Ritenbaugh, F. A. Hubbell, J. Ascensao, R. J. Rodabough, C. A. Rosenberg, V. M. Taylor, R. Harris, C. Chen, L. L. Adams-Campbell, and E. White.** 2004. Estrogen plus progestin and colorectal cancer in postmenopausal women. *N Engl J Med* **350**:991-1004.
50. **Chu, I., K. Blackwell, S. Chen, and J. Slingerland.** 2005. The dual ErbB1/ErbB2 inhibitor, lapatinib (GW572016), cooperates with tamoxifen to inhibit both cell

- proliferation- and estrogen-dependent gene expression in antiestrogen-resistant breast cancer. *Cancer Res* **65**:18-25.
51. **Ciocca, D. R., F. E. Gago, M. A. Fanelli, and S. K. Calderwood.** 2006. Co-expression of steroid receptors (estrogen receptor alpha and/or progesterone receptors) and Her-2/neu: Clinical implications. *J Steroid Biochem Mol Biol* **102**:32-40.
 52. **Coser, K. R., B. S. Wittner, N. F. Rosenthal, S. C. Collins, A. Melas, S. L. Smith, C. J. Mahoney, K. Shioda, K. J. Isselbacher, S. Ramaswamy, and T. Shioda.** 2009. Antiestrogen-resistant subclones of MCF-7 human breast cancer cells are derived from a common monoclonal drug-resistant progenitor. *Proc Natl Acad Sci U S A* **106**:14536-41.
 53. **Couse, J. F., and K. S. Korach.** 1999. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev* **20**:358-417.
 54. **Creighton, C. J., C. Kent Osborne, M. J. van de Vijver, J. A. Foekens, J. G. Klijn, H. M. Horlings, D. Nuyten, Y. Wang, Y. Zhang, G. C. Chamness, S. G. Hilsenbeck, A. V. Lee, and R. Schiff.** 2009. Molecular profiles of progesterone receptor loss in human breast tumors. *Breast Cancer Res Treat* **114**:287-99.
 55. **Cross, A. J., M. F. Leitzmann, M. H. Gail, A. R. Hollenbeck, A. Schatzkin, and R. Sinha.** 2007. A prospective study of red and processed meat intake in relation to cancer risk. *PLoS Med* **4**:e325.
 56. **Cruz-Correa, M., D. A. Shoskes, P. Sanchez, R. Zhao, L. M. Hyland, S. D. Wexner, and F. M. Giardiello.** 2006. Combination treatment with curcumin and quercetin of adenomas in familial adenomatous polyposis. *Clin Gastroenterol Hepatol* **4**:1035-8.
 57. **Cuilliere-Dartigues, P., J. El-Bchiri, A. Krimi, O. Buhard, P. Fontanges, J. F. Flejou, R. Hamelin, and A. Duval.** 2006. TCF-4 isoforms absent in TCF-4 mutated MSI-H colorectal cancer cells colocalize with nuclear CtBP and repress TCF-4-mediated transcription. *Oncogene* **25**:4441-8.
 58. **Cullen, B. R.** 2009. Viral and cellular messenger RNA targets of viral microRNAs. *Nature* **457**:421-5.
 59. **Curigliano, G., and A. Goldhirsch.** 2011. The triple-negative subtype: new ideas for the poorest prognosis breast cancer. *J Natl Cancer Inst Monogr* **2011**:108-10.
 60. **D'Elia, H. F., A. Larsen, L. A. Mattsson, E. Waltbrand, G. Kvist, D. Mellstrom, T. Saxne, C. Ohlsson, E. Nordborg, and H. Carlsten.** 2003. Influence of hormone replacement therapy on disease progression and bone mineral density in rheumatoid arthritis. *J Rheumatol* **30**:1456-63.
 61. **Dahlman-Wright, K., V. Cavailles, S. A. Fuqua, V. C. Jordan, J. A. Katzenellenbogen, K. S. Korach, A. Maggi, M. Muramatsu, M. G. Parker, and J. A. Gustafsson.** 2006. International Union of Pharmacology. LXIV. Estrogen receptors. *Pharmacol Rev* **58**:773-81.
 62. **Dauvois, S., P. S. Danielian, R. White, and M. G. Parker.** 1992. Antiestrogen ICI 164,384 reduces cellular estrogen receptor content by increasing its turnover. *Proc Natl Acad Sci U S A* **89**:4037-41.
 63. **Davies, C., J. Godwin, R. Gray, M. Clarke, D. Cutter, S. Darby, P. McGale, H. C. Pan, C. Taylor, Y. C. Wang, M. Dowsett, J. Ingle, and R. Peto.** 2011. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet* **378**:771-84.
 64. **Davies, R. J., R. Miller, and N. Coleman.** 2005. Colorectal cancer screening: prospects for molecular stool analysis. *Nat Rev Cancer* **5**:199-209.
 65. **Deblois, G., and V. Giguere.** 2003. Ligand-independent coactivation of ERalpha AF-1 by steroid receptor RNA activator (SRA) via MAPK activation. *J Steroid Biochem Mol Biol* **85**:123-31.
 66. **Dews, M., J. L. Fox, S. Hultine, P. Sundaram, W. Wang, Y. Y. Liu, E. Furth, G. H. Enders, W. El-Deiry, J. M. Schelter, M. A. Cleary, and A. Thomas-Tikhonenko.** 2010. The myc-miR-17~92 axis blunts TGF{beta} signaling and production of multiple TGF{beta}-dependent antiangiogenic factors. *Cancer Res* **70**:8233-46.

67. **Duroux-Richard, I., C. Jorgensen, and F. Apparailly.** 2011. What do microRNAs mean for rheumatoid arthritis? *Arthritis Rheum.*
68. **Dykxhoorn, D. M.** 2010. MicroRNAs and metastasis: little RNAs go a long way. *Cancer Res* **70**:6401-6.
69. **Eddy, E. M., T. F. Washburn, D. O. Bunch, E. H. Goulding, B. C. Gladen, D. B. Lubahn, and K. S. Korach.** 1996. Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology* **137**:4796-805.
70. **Edvardsson, K., A. Strom, P. Jonsson, J. A. Gustafsson, and C. Williams.** 2011. Estrogen receptor beta induces antiinflammatory and antitumorigenic networks in colon cancer cells. *Mol Endocrinol* **25**:969-79.
71. **Einspahr, J. G., M. E. Martinez, R. Jiang, C. H. Hsu, A. Rashid, A. K. Bhattacharya, D. J. Ahnen, E. T. Jacobs, P. S. Houlihan, C. R. Webb, D. S. Alberts, and S. R. Hamilton.** 2006. Associations of Ki-ras proto-oncogene mutation and p53 gene overexpression in sporadic colorectal adenomas with demographic and clinicopathologic characteristics. *Cancer Epidemiol Biomarkers Prev* **15**:1443-50.
72. **Ekbom, A., C. Helmick, M. Zack, and H. O. Adami.** 1990. Increased risk of large-bowel cancer in Crohn's disease with colonic involvement. *Lancet* **336**:357-9.
73. **Elvidge, G.** 2006. Microarray expression technology: from start to finish. *Pharmacogenomics* **7**:123-34.
74. **Emde, A., G. Mahlknecht, K. Maslak, B. Ribba, M. Sela, K. Possinger, and Y. Yarden.** 2011. Simultaneous Inhibition of Estrogen Receptor and the HER2 Pathway in Breast Cancer: Effects of HER2 Abundance. *Transl Oncol* **4**:293-300.
75. **Enmark, E., M. Peltö-Huikko, K. Grandien, S. Lagercrantz, J. Lagercrantz, G. Fried, M. Nordenskjöld, and J. A. Gustafsson.** 1997. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J Clin Endocrinol Metab* **82**:4258-65.
76. **Esslimani-Sahla, M., A. Kramar, J. Simony-Lafontaine, M. Warner, J. A. Gustafsson, and H. Rochefort.** 2005. Increased estrogen receptor [luc]beta[rcub]cx expression during mammary carcinogenesis. *Clin Cancer Res* **11**:3170-3174.
77. **Evans, A. H., S. Pancholi, I. Farmer, A. Thornhill, D. B. Evans, S. R. Johnston, M. Dowsett, and L. A. Martin.** 2010. EGFR/HER2 inhibitor AEE788 increases ER-mediated transcription in HER2/ER-positive breast cancer cells but functions synergistically with endocrine therapy. *Br J Cancer* **102**:1235-43.
78. **Fearon, E. R.** 2011. Molecular genetics of colorectal cancer. *Annu Rev Pathol* **6**:479-507.
79. **Fearon, E. R., and B. Vogelstein.** 1990. A genetic model for colorectal tumorigenesis. *Cell* **61**:759-67.
80. **Feng, Y., D. Manka, K. U. Wagner, and S. A. Khan.** 2007. Estrogen receptor-alpha expression in the mammary epithelium is required for ductal and alveolar morphogenesis in mice. *Proc Natl Acad Sci U S A* **104**:14718-23.
81. **Ferlay J, S. H., Bray F, Forman D, Mathers C and Parkin DM,** posting date. GLOBOCAN 2008 v1.2, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet] Lyon, France: International Agency for Research on Cancer; 2010. Available from: <http://globocan.iarc.fr>, accessed on 22/10/11.
82. **Fiorelli, G., L. Picariello, V. Martinetti, F. Tonelli, and M. L. Brandi.** 1999. Functional estrogen receptor beta in colon cancer cells. *Biochem Biophys Res Commun* **261**:521-7.
83. **Fisher, B., J. P. Costantino, D. L. Wickerham, R. S. Cecchini, W. M. Cronin, A. Robidoux, T. B. Bevers, M. T. Kavanah, J. N. Atkins, R. G. Margolese, C. D. Runowicz, J. M. James, L. G. Ford, and N. Wolmark.** 2005. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Natl Cancer Inst* **97**:1652-62.
84. **Fisher, B., J. P. Costantino, D. L. Wickerham, C. K. Redmond, M. Kavanah, W. M. Cronin, V. Vogel, A. Robidoux, N. Dimitrov, J. Atkins, M. Daly, S. Wieand, E. Tan-Chiu, L. Ford, and N. Wolmark.** 1998. Tamoxifen for prevention of breast

- cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* **90**:1371-88.
85. **Foley, E. F., A. A. Jazaeri, M. A. Shupnik, O. Jazaeri, and L. W. Rice.** 2000. Selective loss of estrogen receptor beta in malignant human colon. *Cancer Res* **60**:245-8.
 86. **Forster, C., S. Makela, A. Warri, S. Kietz, D. Becker, K. Hultenby, M. Warner, and J. A. Gustafsson.** 2002. Involvement of estrogen receptor beta in terminal differentiation of mammary gland epithelium. *Proc Natl Acad Sci U S A* **99**:15578-83.
 87. **Fox, E. M., R. J. Davis, and M. A. Shupnik.** 2008. ERbeta in breast cancer--onlooker, passive player, or active protector? *Steroids* **73**:1039-51.
 88. **Frasor, J., E. C. Chang, B. Komm, C. Y. Lin, V. B. Vega, E. T. Liu, L. D. Miller, J. Smeds, J. Bergh, and B. S. Katzenellenbogen.** 2006. Gene expression preferentially regulated by tamoxifen in breast cancer cells and correlations with clinical outcome. *Cancer Res* **66**:7334-40.
 89. **Frasor, J., J. M. Danes, B. Komm, K. C. N. Chang, C. R. Lyttle, and B. S. Katzenellenbogen.** 2003. Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. *Endocrinology* **144**:4562-4574.
 90. **Frasor, J., A. Weaver, M. Pradhan, Y. Dai, L. D. Miller, C. Y. Lin, and A. Stanculescu.** 2009. Positive cross-talk between estrogen receptor and NF-kappaB in breast cancer. *Cancer Res* **69**:8918-25.
 91. **Friedman, R. C., K. K. Farh, C. B. Burge, and D. P. Bartel.** 2009. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* **19**:92-105.
 92. **Germain, P., B. Staels, C. Dacquet, M. Spedding, and V. Laudet.** 2006. Overview of nomenclature of nuclear receptors. *Pharmacol Rev* **58**:685-704.
 93. **Giroux, V., G. Bernatchez, and J. C. Carrier.** 2010. Chemopreventive effect of ERbeta-Selective agonist on intestinal tumorigenesis in Apc(Min/+) Mice. *Mol Carcinog*:DOI: 10.1002/mc.20719.
 94. **Giroux, V., F. Lemay, G. Bernatchez, Y. Robitaille, and J. C. Carrier.** 2008. Estrogen receptor beta deficiency enhances small intestinal tumorigenesis in ApcMin/+ mice. *Int J Cancer* **123**:303-11.
 95. **Gold, E. B.** 2011. The timing of the age at which natural menopause occurs. *Obstet Gynecol Clin North Am* **38**:425-40.
 96. **Goss, K. H., and J. Groden.** 2000. Biology of the adenomatous polyposis coli tumor suppressor. *J Clin Oncol* **18**:1967-79.
 97. **Green, S., P. Walter, V. Kumar, A. Krust, J. M. Bornert, P. Argos, and P. Chambon.** 1986. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* **320**:134-9.
 98. **Greene, G. L., P. Gilna, M. Waterfield, A. Baker, Y. Hort, and J. Shine.** 1986. Sequence and expression of human estrogen receptor complementary DNA. *Science* **231**:1150-4.
 99. **Grivas, P. D., V. Tzelepi, G. Sotiropoulou-Bonikou, Z. Kefalopoulou, A. G. Papavassiliou, and H. Kalofonos.** 2009. Estrogen receptor alpha/beta, AIB1, and TIF2 in colorectal carcinogenesis: do coregulators have prognostic significance? *Int J Colorectal Dis* **24**:613-22.
 100. **Grober, O. M., M. Mutarelli, G. Giurato, M. Ravo, L. Cicatiello, M. R. De Filippo, L. Ferraro, G. Nassa, M. F. Papa, O. Paris, R. Tarallo, S. Luo, G. P. Schroth, V. Benes, and A. Weisz.** 2011. Global analysis of estrogen receptor beta binding to breast cancer cell genome reveals an extensive interplay with estrogen receptor alpha for target gene regulation. *BMC Genomics* **12**:36.
 101. **Groden, J., A. Thliveris, W. Samowitz, M. Carlson, L. Gelbert, H. Albertsen, G. Joslyn, J. Stevens, L. Spirio, M. Robertson, and et al.** 1991. Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* **66**:589-600.
 102. **Grodstein, F., P. A. Newcomb, and M. J. Stampfer.** 1999. Postmenopausal hormone therapy and the risk of colorectal cancer: a review and meta-analysis. *Am J Med* **106**:574-82.

103. **Guay, C., E. Roggli, V. Nesca, C. Jacovetti, and R. Regazzi.** 2011. Diabetes mellitus, a microRNA-related disease? *Transl Res* **157**:253-64.
104. **Guenther, M. G., O. Barak, and M. A. Lazar.** 2001. The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3. *Mol Cell Biol* **21**:6091-101.
105. **Gustafsson, J.** 2006. ER β scientific visions translate to clinical uses. *Climacteric* **9**:156-160.
106. **Guzeloglu-Kayisli, O., G. Halis, S. Taskiran, U. A. Kayisli, and A. Arici.** 2008. DNA-binding ability of NF-kappaB is affected differently by ERalpha and ERbeta and its activation results in inhibition of estrogen responsiveness. *Reprod Sci* **15**:493-505.
107. **Ha, T. Y.** 2011. MicroRNAs in Human Diseases: From Cancer to Cardiovascular Disease. *Immune Netw* **11**:135-54.
108. **Hall, J. M., and D. P. McDonnell.** 2005. Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. *Mol Interv* **5**:343-57.
109. **Harada, N., Y. Tamai, T. Ishikawa, B. Sauer, K. Takaku, M. Oshima, and M. M. Taketo.** 1999. Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *EMBO J* **18**:5931-42.
110. **Harnish, D. C., M. S. Scicchitano, S. J. Adelman, C. R. Lyttle, and S. K. Karathanasis.** 2000. The role of CBP in estrogen receptor cross-talk with nuclear factor-kappaB in HepG2 cells. *Endocrinology* **141**:3403-11.
111. **Hartman, H. B., J. Yu, T. Alenghat, T. Ishizuka, and M. A. Lazar.** 2005. The histone-binding code of nuclear receptor co-repressors matches the substrate specificity of histone deacetylase 3. *EMBO Rep* **6**:445-51.
112. **Hartman, J., K. Edvardsson, K. Lindberg, C. Zhao, C. Williams, A. Strom, and J. A. Gustafsson.** 2009. Tumor repressive functions of estrogen receptor beta in SW480 colon cancer cells. *Cancer Res* **69**:6100-6.
113. **Hartman, J., K. Lindberg, A. Morani, J. Inzunza, A. Strom, and J. A. Gustafsson.** 2006. Estrogen receptor beta inhibits angiogenesis and growth of T47D breast cancer xenografts. *Cancer Res* **66**:11207-13.
114. **Harvey, J. M., G. M. Clark, C. K. Osborne, and D. C. Allred.** 1999. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol* **17**:1474-81.
115. **He, L., and G. J. Hannon.** 2004. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* **5**:522-31.
116. **He, L., J. M. Thomson, M. T. Hemann, E. Hernando-Monge, D. Mu, S. Goodson, S. Powers, C. Cordon-Cardo, S. W. Lowe, G. J. Hannon, and S. M. Hammond.** 2005. A microRNA polycistron as a potential human oncogene. *Nature* **435**:828-33.
117. **Heldin, C. H., and A. Moustakas.** 2011. Role of Smads in TGFbeta signaling. *Cell Tissue Res*.
118. **Heldring, N., A. Pike, S. Andersson, J. Matthews, G. Cheng, J. Hartman, M. Tujague, A. Strom, E. Treuter, M. Warner, and J. A. Gustafsson.** 2007. Estrogen receptors: how do they signal and what are their targets. *Physiol Rev* **87**:905-31.
119. **Hendifar, A., D. Yang, F. Lenz, G. Lurje, A. Pohl, C. Lenz, Y. Ning, W. Zhang, and H. J. Lenz.** 2009. Gender disparities in metastatic colorectal cancer survival. *Clin Cancer Res* **15**:6391-7.
120. **Hennighausen, L., and G. W. Robinson.** 2005. Information networks in the mammary gland. *Nat Rev Mol Cell Biol* **6**:715-25.
121. **Hennighausen, L., and G. W. Robinson.** 2001. Signaling pathways in mammary gland development. *Dev Cell* **1**:467-75.
122. **Hopp, T. A., H. L. Weiss, I. S. Parra, Y. Cui, C. K. Osborne, and S. A. Fuqua.** 2004. Low levels of estrogen receptor beta protein predict resistance to tamoxifen therapy in breast cancer. *Clin Cancer Res* **10**:7490-9.
123. **Hou, Y. F., S. T. Yuan, H. C. Li, J. Wu, J. S. Lu, G. Liu, L. J. Lu, Z. Z. Shen, J. Ding, and Z. M. Shao.** 2004. ERbeta exerts multiple stimulative effects on human breast carcinoma cells. *Oncogene* **23**:5799-806.

124. **Hua, S., C. B. Kallen, R. Dhar, M. T. Baquero, C. E. Mason, B. A. Russell, P. K. Shah, J. Liu, A. Khramtsov, M. S. Tretiakova, T. N. Krausz, O. I. Olopade, D. L. Rimm, and K. P. White.** 2008. Genomic analysis of estrogen cascade reveals histone variant H2A.Z associated with breast cancer progression. *Mol Syst Biol* **4**:188.
125. **Hua, Y. J., K. Tu, Z. Y. Tang, Y. X. Li, and H. S. Xiao.** 2008. Comparison of normalization methods with microRNA microarray. *Genomics* **92**:122-8.
126. **Huang, P., V. Chandra, and F. Rastinejad.** 2010. Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics. *Annu Rev Physiol* **72**:247-72.
127. **Hue-Roye, K., and S. Vege.** 2008. Principles of PCR-based assays. *Immunohematology* **24**:170-5.
128. **Hurvitz, S. A., and R. J. Pietras.** 2008. Rational management of endocrine resistance in breast cancer: a comprehensive review of estrogen receptor biology, treatment options, and future directions. *Cancer* **113**:2385-97.
129. **Iorio, M. V., M. Ferracin, C. G. Liu, A. Veronese, R. Spizzo, S. Sabbioni, E. Magri, M. Pedriali, M. Fabbri, M. Campiglio, S. Menard, J. P. Palazzo, A. Rosenberg, P. Musiani, S. Volinia, I. Nenci, G. A. Calin, P. Querzoli, M. Negrini, and C. M. Croce.** 2005. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res* **65**:7065-70.
130. **Jacobs, E. J., E. White, and N. S. Weiss.** 1994. Exogenous hormones, reproductive history, and colon cancer (Seattle, Washington, USA). *Cancer Causes Control* **5**:359-66.
131. **Jassam, N., S. M. Bell, V. Speirs, and P. Quirke.** 2005. Loss of expression of oestrogen receptor beta in colon cancer and its association with Dukes' staging. *Oncol Rep* **14**:17-21.
132. **Jensen, E. V.** 1962. On the mechanism of estrogen action. *Perspect Biol Med* **6**:47-59.
133. **Johnson, C. D., A. Esquela-Kerscher, G. Stefani, M. Byrom, K. Kelnar, D. Ovcharenko, M. Wilson, X. Wang, J. Shelton, J. Shingara, L. Chin, D. Brown, and F. J. Slack.** 2007. The let-7 microRNA represses cell proliferation pathways in human cells. *Cancer Res* **67**:7713-22.
134. **Jones, M. E., W. C. Boon, K. McInnes, L. Maffei, C. Carani, and E. R. Simpson.** 2007. Recognizing rare disorders: aromatase deficiency. *Nat Clin Pract Endocrinol Metab* **3**:414-21.
135. **Joshi, P. A., H. W. Jackson, A. G. Beristain, M. A. Di Grappa, P. A. Mote, C. L. Clarke, J. Stingl, P. D. Waterhouse, and R. Khokha.** 2010. Progesterone induces adult mammary stem cell expansion. *Nature* **465**:803-7.
136. **Joslyn, G., M. Carlson, A. Thliveris, H. Albertsen, L. Gelbert, W. Samowitz, J. Groden, J. Stevens, L. Spirio, M. Robertson, and et al.** 1991. Identification of deletion mutations and three new genes at the familial polyposis locus. *Cell* **66**:601-13.
137. **Katchy, A., K. Edvardsson, E. Aydogdu, and C. Williams.** 2011. Estradiol-activated estrogen receptor α does not regulate mature microRNAs in T47D breast cancer cells. *J Steroid Biochem Mol Biol*, doi:10.1016/j.jsbmb.2011.10.008.
138. **Kato, S., Y. Masuhiro, M. Watanabe, Y. Kobayashi, K. I. Takeyama, H. Endoh, and J. Yanagisawa.** 2000. Molecular mechanism of a cross-talk between oestrogen and growth factor signalling pathways. *Genes Cells* **5**:593-601.
139. **Kennelly, R., D. O. Kavanagh, A. M. Hogan, and D. C. Winter.** 2008. Oestrogen and the colon: potential mechanisms for cancer prevention. *Lancet Oncol* **9**:385-91.
140. **Kim, J. W., S. Mori, and J. R. Nevins.** 2010. Myc-induced microRNAs integrate Myc-mediated cell proliferation and cell fate. *Cancer Res* **70**:4820-8.
141. **Kininis, M., B. S. Chen, A. G. Diehl, G. D. Isaacs, T. Zhang, A. C. Siepel, A. G. Clark, and W. L. Kraus.** 2007. Genomic analyses of transcription factor binding, histone acetylation, and gene expression reveal mechanistically distinct classes of estrogen-regulated promoters. *Mol Cell Biol* **27**:5090-104.
142. **Kinzler, K. W., and B. Vogelstein.** 1996. Lessons from hereditary colorectal cancer. *Cell* **87**:159-70.

143. **Knight, W. A., R. B. Livingston, E. J. Gregory, and W. L. McGuire.** 1977. Estrogen receptor as an independent prognostic factor for early recurrence in breast cancer. *Cancer Res* **37**:4669-71.
144. **Konstantinopoulos, P. A., A. Kominea, G. Vondoros, G. P. Sykiotis, P. Andricopoulos, I. Varakis, G. Sotiropoulou-Bonikou, and A. G. Papavassiliou.** 2003. Oestrogen receptor beta (ERbeta) is abundantly expressed in normal colonic mucosa, but declines in colon adenocarcinoma paralleling the tumour's dedifferentiation. *Eur J Cancer* **39**:1251-8.
145. **Koo, J. H., B. Jalaludin, S. K. Wong, A. Kneebone, S. J. Connor, and R. W. Leong.** 2008. Improved survival in young women with colorectal cancer. *Am J Gastroenterol* **103**:1488-95.
146. **Korach, K. S.** 1994. Insights from the study of animals lacking functional estrogen receptor. *Science* **266**:1524-7.
147. **Koushik, A., D. J. Hunter, D. Spiegelman, W. L. Beeson, P. A. van den Brandt, J. E. Buring, E. E. Calle, E. Cho, G. E. Fraser, J. L. Freudenheim, C. S. Fuchs, E. L. Giovannucci, R. A. Goldbohm, L. Harnack, D. R. Jacobs, Jr., I. Kato, V. Krogh, S. C. Larsson, M. F. Leitzmann, J. R. Marshall, M. L. McCullough, A. B. Miller, P. Pietinen, T. E. Rohan, A. Schatzkin, S. Sieri, M. J. Virtanen, A. Wolk, A. Zeleniuch-Jacquotte, S. M. Zhang, and S. A. Smith-Warner.** 2007. Fruits, vegetables, and colon cancer risk in a pooled analysis of 14 cohort studies. *J Natl Cancer Inst* **99**:1471-83.
148. **Kronenberg, H., S. Memed, K. Polonsky, and R. Larsen.** 2007. *Williams Textbook of Endocrinology*, 11 ed. Saunders.
149. **Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller.** 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**:263-74.
150. **Kuijsten, A., I. C. Arts, P. C. Hollman, P. van't Veer, and E. Kampman.** 2006. Plasma enterolignans are associated with lower colorectal adenoma risk. *Cancer Epidemiol Biomarkers Prev* **15**:1132-6.
151. **Kuiper, G. G., E. Enmark, M. Peltö-Huikko, S. Nilsson, and J. A. Gustafsson.** 1996. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A* **93**:5925-30.
152. **Langholz, E., P. Munkholm, M. Davidsen, and V. Binder.** 1992. Colorectal cancer risk and mortality in patients with ulcerative colitis. *Gastroenterology* **103**:1444-51.
153. **Lavinsky, R. M., K. Jepsen, T. Heinzel, J. Torchia, T. M. Mullen, R. Schiff, A. L. Del-Rio, M. Ricote, S. Ngo, J. Gemsch, S. G. Hilsenbeck, C. K. Osborne, C. K. Glass, M. G. Rosenfeld, and D. W. Rose.** 1998. Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc Natl Acad Sci U S A* **95**:2920-5.
154. **Le Quesne, J., and C. Caldas.** Micro-RNAs and breast cancer. *Mol Oncol* **4**:230-41.
155. **Lee, R. C., R. L. Feinbaum, and V. Ambros.** 1993. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* **75**:843-54.
156. **Leo, C., H. Li, and J. D. Chen.** 2000. Differential mechanisms of nuclear receptor regulation by receptor-associated coactivator 3. *J Biol Chem* **275**:5976-82.
157. **Levy, N., S. Paruthiyil, X. Zhao, O. I. Vivar, E. F. Saunier, C. Griffin, M. Tagliaferri, I. Cohen, T. P. Speed, and D. C. Leitman.** 10. Unliganded estrogen receptor-beta regulation of genes is inhibited by tamoxifen. *Mol Cell Endocrinol* **315**:201-7.
158. **Li, X., S. U. Mertens-Talcott, S. Zhang, K. Kim, J. Ball, and S. Safe.** 2010. MicroRNA-27a Indirectly Regulates Estrogen Receptor {alpha} Expression and Hormone Responsiveness in MCF-7 Breast Cancer Cells. *Endocrinology* **151**:2462-73.
159. **Li, Y., M. H. Lambert, and H. E. Xu.** 2003. Activation of nuclear receptors: a perspective from structural genomics. *Structure* **11**:741-6.
160. **Lin, C. Y., V. B. Vega, J. S. Thomsen, T. Zhang, S. L. Kong, M. Xie, K. P. Chiu, L. Lipovich, D. H. Barnett, F. Stossi, A. Yeo, J. George, V. A. Kuznetsov, Y. K. Lee, T. H. Charn, N. Palanisamy, L. D. Miller, E. Cheung, B. S. Katzenellenbogen, Y.**

- Ruan, G. Bourque, C. L. Wei, and E. T. Liu. 2007. Whole-genome cartography of estrogen receptor alpha binding sites. *PLoS Genet* **3**:e87.
161. Lin, J., S. M. Zhang, N. R. Cook, J. E. Manson, J. E. Buring, and I. M. Lee. 2007. Oral contraceptives, reproductive factors, and risk of colorectal cancer among women in a prospective cohort study. *Am J Epidemiol* **165**:794-801.
162. Lin, S., W. K. Cheung, S. Chen, G. Lu, Z. Wang, D. Xie, K. Li, M. C. Lin, and H. F. Kung. 2010. Computational identification and characterization of primate-specific microRNAs in human genome. *Comput Biol Chem* **34**:232-41.
163. Lindberg, K., L. A. Helguero, Y. Omoto, J. A. Gustafsson, and L. A. Haldosen. 2011. Estrogen receptor beta represses Akt signaling in breast cancer cells via downregulation of HER2/HER3 and upregulation of PTEN: implications for tamoxifen sensitivity. *Breast Cancer Res* **13**:R43.
164. Locker, G. Y. 1998. Hormonal therapy of breast cancer. *Cancer Treat Rev* **24**:221-40.
165. Long, M. D., C. F. Martin, J. A. Galanko, and R. S. Sandler. 2010. Hormone replacement therapy, oral contraceptive use, and distal large bowel cancer: a population-based case-control study. *Am J Gastroenterol* **105**:1843-50.
166. Lu, B., J. Xu, J. Chen, J. Yu, E. Xu, and M. Lai. 2008. TaqMan low density array is roughly right for gene expression quantification in colorectal cancer. *Clin Chim Acta* **389**:146-51.
167. Lu, W. J., Z. Desta, and D. A. Flockhart. 2011. Tamoxifen metabolites as active inhibitors of aromatase in the treatment of breast cancer. *Breast Cancer Res Treat*.
168. Malone, J. H., and B. Oliver. 2011. Microarrays, deep sequencing and the true measure of the transcriptome. *BMC Biol* **9**:34.
169. Mangelsdorf, D. J., C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, and R. M. Evans. 1995. The nuclear receptor superfamily: the second decade. *Cell* **83**:835-9.
170. Markowitz, S. D., and M. M. Bertagnolli. 2009. Molecular origins of cancer: Molecular basis of colorectal cancer. *N Engl J Med* **361**:2449-60.
171. Marques-Vidal, P., P. Ravasco, and M. Ermelinda Camilo. 2006. Foodstuffs and colorectal cancer risk: a review. *Clin Nutr* **25**:14-36.
172. Martineti, V., L. Picariello, I. Tognarini, S. Carbonell Sala, A. Gozzini, C. Azzari, C. Mavilia, A. Tanini, A. Falchetti, G. Fiorelli, F. Tonelli, and M. L. Brandi. 2005. ERbeta is a potent inhibitor of cell proliferation in the HCT8 human colon cancer cell line through regulation of cell cycle components. *Endocr Relat Cancer* **12**:455-69.
173. Maruvada, P., C. T. Baumann, G. L. Hager, and P. M. Yen. 2003. Dynamic shuttling and intranuclear mobility of nuclear hormone receptors. *J Biol Chem* **278**:12425-32.
174. Massarweh, S., C. K. Osborne, C. J. Creighton, L. Qin, A. Tsimelzon, S. Huang, H. Weiss, M. Rimawi, and R. Schiff. 2008. Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. *Cancer Res* **68**:826-33.
175. Matthews, J., and J. A. Gustafsson. 2003. Estrogen signaling: a subtle balance between ER alpha and ER beta. *Mol Interv* **3**:281-92.
176. Mattie, M. D., C. C. Benz, J. Bowers, K. Sensinger, L. Wong, G. K. Scott, V. Fedele, D. Ginzinger, R. Getts, and C. Haqq. 2006. Optimized high-throughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies. *Mol Cancer* **5**:24.
177. Mavaddat, N., A. C. Antoniou, D. F. Easton, and M. Garcia-Closas. 2010. Genetic susceptibility to breast cancer. *Mol Oncol* **4**:174-91.
178. Michael, M. Z., O. C. SM, N. G. van Holst Pellekaan, G. P. Young, and R. J. James. 2003. Reduced accumulation of specific microRNAs in colorectal neoplasia. *Mol Cancer Res* **1**:882-91.
179. Mueller, S. O., S. Simon, K. Chae, M. Metzler, and K. S. Korach. 2004. Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor alpha (ERalpha) and ERbeta in human cells. *Toxicol Sci* **80**:14-25.

180. **Munkholm, P.** 2003. Review article: the incidence and prevalence of colorectal cancer in inflammatory bowel disease. *Aliment Pharmacol Ther* **18 Suppl 2**:1-5.
181. **Murphy, L. C., S. V. Seekallu, and P. H. Watson.** 2011. Clinical significance of estrogen receptor phosphorylation. *Endocr Relat Cancer* **18**:R1-14.
182. **Newcomb, P. A., and B. E. Storer.** 1995. Postmenopausal hormone use and risk of large-bowel cancer. *J Natl Cancer Inst* **87**:1067-71.
183. **Nilsson, S., S. Makela, E. Treuter, M. Tujague, J. Thomsen, G. Andersson, E. Enmark, K. Pettersson, M. Warner, and J. A. Gustafsson.** 2001. Mechanisms of estrogen action. *Physiol Rev* **81**:1535-65.
184. **Nugent, M., N. Miller, and M. J. Kerin.** 2011. MicroRNAs in colorectal cancer: function, dysregulation and potential as novel biomarkers. *Eur J Surg Oncol* **37**:649-54.
185. **Nussler, N. C., K. Reinbacher, N. Shanny, A. Schirmeier, M. Glanemann, P. Neuhaus, A. K. Nussler, and M. Kirschner.** 2008. Sex-specific differences in the expression levels of estrogen receptor subtypes in colorectal cancer. *Gend Med* **5**:209-17.
186. **O'Donnell, K. A., E. A. Wentzel, K. I. Zeller, C. V. Dang, and J. T. Mendell.** 2005. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* **435**:839-43.
187. **Obiorah, I., and V. C. Jordan.** 2011. Progress in endocrine approaches to the treatment and prevention of breast cancer. *Maturitas*.
188. **Olivo-Marston, S. E., L. E. Mechanic, S. Mollerup, E. D. Bowman, A. T. Remaley, M. R. Forman, V. Skaug, Y. L. Zheng, A. Haugen, and C. C. Harris.** 2010. Serum estrogen and tumor-positive estrogen receptor-alpha are strong prognostic classifiers of non-small-cell lung cancer survival in both men and women. *Carcinogenesis* **31**:1778-86.
189. **Osborne, C. K.** 1998. Steroid hormone receptors in breast cancer management. *Breast Cancer Res Treat* **51**:227-38.
190. **Osborne, C. K., and R. Schiff.** Mechanisms of endocrine resistance in breast cancer. *Annu Rev Med* **62**:233-47.
191. **Oshima, M., H. Oshima, K. Kitagawa, M. Kobayashi, C. Itakura, and M. Taketo.** 1995. Loss of Apc heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated Apc gene. *Proc Natl Acad Sci U S A* **92**:4482-6.
192. **Ouyang, W., S. Rutz, N. K. Crellin, P. A. Valdez, and S. G. Hymowitz.** 2011. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol* **29**:71-109.
193. **Paech, K., P. Webb, G. G. Kuiper, S. Nilsson, J. Gustafsson, P. J. Kushner, and T. S. Scanlan.** 1997. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* **277**:1508-10.
194. **Palmieri, C., G. J. Cheng, S. Saji, M. Zelada-Hedman, A. Warri, Z. Weihua, S. Van Noorden, T. Wahlstrom, R. C. Coombes, M. Warner, and J. A. Gustafsson.** 2002. Estrogen receptor beta in breast cancer. *Endocr Relat Cancer* **9**:1-13.
195. **Pandey, D. P., and D. Picard.** 2009. miR-22 inhibits estrogen signaling by directly targeting the estrogen receptor alpha mRNA. *Mol Cell Biol* **29**:3783-90.
196. **Papoutsis, Z., C. Zhao, M. Putnik, J. A. Gustafsson, and K. Dahlman-Wright.** 2009. Binding of estrogen receptor alpha/beta heterodimers to chromatin in MCF-7 cells. *J Mol Endocrinol* **43**:65-72.
197. **Park, B. W., K. S. Kim, M. K. Heo, S. S. Ko, S. W. Hong, W. I. Yang, J. H. Kim, G. E. Kim, and K. S. Lee.** 2003. Expression of estrogen receptor-beta in normal mammary and tumor tissues: is it protective in breast carcinogenesis? *Breast Cancer Res Treat* **80**:79-85.
198. **Pasquinelli, A. E., B. J. Reinhart, F. Slack, M. Q. Martindale, M. I. Kuroda, B. Maller, D. C. Hayward, E. E. Ball, B. Degnan, P. Muller, J. Spring, A. Srinivasan, M. Fishman, J. Finnerty, J. Corbo, M. Levine, P. Leahy, E. Davidson, and G. Ruvkun.** 2000. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* **408**:86-9.
199. **Petrova, T. V., A. Nykanen, C. Norrmen, K. I. Ivanov, L. C. Andersson, C. Haglund, P. Puolakkainen, F. Wempe, H. von Melchner, G. Gradwohl, S. Vanharanta, L. A. Aaltonen, J. Saharinen, M. Gentile, A. Clarke, J. Taipale, G.**

- Oliver, and K. Alitalo.** 2008. Transcription factor PROX1 induces colon cancer progression by promoting the transition from benign to highly dysplastic phenotype. *Cancer Cell* **13**:407-19.
200. **Pettersson, K., K. Grandien, G. G. Kuiper, and J. A. Gustafsson.** 1997. Mouse estrogen receptor beta forms estrogen response element-binding heterodimers with estrogen receptor alpha. *Mol Endocrinol* **11**:1486-96.
201. **Pettersson, K., and J. A. Gustafsson.** 2001. Role of estrogen receptor beta in estrogen action. *Annu Rev Physiol* **63**:165-92.
202. **Platet, N., A. M. Cathiard, M. Gleizes, and M. Garcia.** 2004. Estrogens and their receptors in breast cancer progression: a dual role in cancer proliferation and invasion. *Crit Rev Oncol Hematol* **51**:55-67.
203. **Polakis, P.** 2007. The many ways of Wnt in cancer. *Curr Opin Genet Dev* **17**:45-51.
204. **Popivanova, B. K., K. Kitamura, Y. Wu, T. Kondo, T. Kagaya, S. Kaneko, M. Oshima, C. Fujii, and N. Mukaida.** 2008. Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis. *J Clin Invest* **118**:560-70.
205. **Pourcet, B., I. Pineda-Torra, B. Derudas, B. Staels, and C. Glineur.** 2010. SUMOylation of human peroxisome proliferator-activated receptor alpha inhibits its trans-activity through the recruitment of the nuclear corepressor NCoR. *J Biol Chem* **285**:5983-92.
206. **Pradervand, S., J. Weber, J. Thomas, M. Bueno, P. Wirapati, K. Lefort, G. P. Dotto, and K. Harshman.** 2009. Impact of normalization on miRNA microarray expression profiling. *RNA* **15**:493-501.
207. **Raju, J., A. Bielecki, D. Caldwell, E. Lok, M. Taylor, K. Kapal, I. Curran, G. M. Cooke, R. P. Bird, and R. Mehta.** 2009. Soy isoflavones modulate azoxymethane-induced rat colon carcinogenesis exposed pre- and postnatally and inhibit growth of DLD-1 human colon adenocarcinoma cells by increasing the expression of estrogen receptor-beta. *J Nutr* **139**:474-81.
208. **Reinhart, B. J., F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger, A. E. Rougvie, H. R. Horvitz, and G. Ruvkun.** 2000. The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* **403**:901-6.
209. **Rich, B. E., and T. S. Kupper.** 2001. Cytokines: IL-20 - a new effector in skin inflammation. *Curr Biol* **11**:R531-4.
210. **Richert, M. M., K. L. Schwertfeger, J. W. Ryder, and S. M. Anderson.** 2000. An atlas of mouse mammary gland development. *J Mammary Gland Biol Neoplasia* **5**:227-41.
211. **Riggins, R. B., R. S. Schrecengost, M. S. Guerrero, and A. H. Bouton.** 2007. Pathways to tamoxifen resistance. *Cancer Lett* **256**:1-24.
212. **Rizzo, A., F. Pallone, G. Monteleone, and M. C. Fantini.** 2011. Intestinal inflammation and colorectal cancer: a double-edged sword? *World J Gastroenterol* **17**:3092-100.
213. **Robertson, K. M., L. O'Donnell, M. E. Jones, S. J. Meachem, W. C. Boon, C. R. Fisher, K. H. Graves, R. I. McLachlan, and E. R. Simpson.** 1999. Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. *Proc Natl Acad Sci U S A* **96**:7986-91.
214. **Ross-Innes, C. S., R. Stark, K. A. Holmes, D. Schmidt, C. Spyrou, R. Russell, C. E. Massie, S. L. Vowler, M. Eldridge, and J. S. Carroll.** 2010. Cooperative interaction between retinoic acid receptor-alpha and estrogen receptor in breast cancer. *Genes Dev* **24**:171-82.
215. **Schena, M., R. A. Heller, T. P. Theriault, K. Konrad, E. Lachenmeier, and R. W. Davis.** 1998. Microarrays: biotechnology's discovery platform for functional genomics. *Trends Biotechnol* **16**:301-6.
216. **Schena, M., D. Shalon, R. W. Davis, and P. O. Brown.** 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**:467-70.
217. **Segditsas, S., and I. Tomlinson.** 2006. Colorectal cancer and genetic alterations in the Wnt pathway. *Oncogene* **25**:7531-7.

218. **Shiau, A. K., D. Barstad, P. M. Loria, L. Cheng, P. J. Kushner, D. A. Agard, and G. L. Greene.** 1998. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95**:927-37.
219. **Shou, J., S. Massarweh, C. K. Osborne, A. E. Wakeling, S. Ali, H. Weiss, and R. Schiff.** 2004. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. *J Natl Cancer Inst* **96**:926-35.
220. **Siegel, R., E. Ward, O. Brawley, and A. Jemal.** 2011. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J Clin* **61**:212-36.
221. **Simpson, E. R.** 2003. Sources of estrogen and their importance. *J Steroid Biochem Mol Biol* **86**:225-30.
222. **Simpson, E. R., C. Clyne, G. Rubin, W. C. Boon, K. Robertson, K. Britt, C. Speed, and M. Jones.** 2002. Aromatase--a brief overview. *Annu Rev Physiol* **64**:93-127.
223. **Skloris, G. P., Z. J. Nugent, B. G. Rowan, C. R. Penner, P. H. Watson, and L. C. Murphy.** 2010. A phosphorylation code for oestrogen receptor-alpha predicts clinical outcome to endocrine therapy in breast cancer. *Endocr Relat Cancer* **17**:589-97.
224. **Skog, M., P. Bono, M. Lundin, J. Lundin, J. Louhimo, N. Linder, T. V. Petrova, L. C. Andersson, H. Joensuu, K. Alitalo, and C. H. Haglund.** 2011. Expression and prognostic value of transcription factor PROX1 in colorectal cancer. *Br J Cancer* **105**:1346-51.
225. **Slamon, D. J., W. Godolphin, L. A. Jones, J. A. Holt, S. G. Wong, D. E. Keith, W. J. Levin, S. G. Stuart, J. Udove, A. Ullrich, and et al.** 1989. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**:707-12.
226. **Slattery, M. L., W. S. Samowitz, and J. A. Holden.** 2000. Estrogen and progesterone receptors in colon tumors. *Am J Clin Pathol* **113**:364-8.
227. **Smirnoff, P., Y. Liel, J. Gnainsky, S. Shany, and B. Schwartz.** 1999. The protective effect of estrogen against chemically induced murine colon carcinogenesis is associated with decreased CpG island methylation and increased mRNA and protein expression of the colonic vitamin D receptor. *Oncol Res* **11**:255-64.
228. **Spizzo, R., M. S. Nicoloso, L. Lupini, Y. Lu, J. Fogarty, S. Rossi, B. Zagatti, M. Fabbri, A. Veronese, X. Liu, R. Davuluri, C. M. Croce, G. Mills, M. Negrini, and G. A. Calin.** 2010. miR-145 participates with TP53 in a death-promoting regulatory loop and targets estrogen receptor-alpha in human breast cancer cells. *Cell Death Differ* **17**:246-54.
229. **Stoica, G. E., T. F. Franke, A. Wellstein, F. Czubayko, H. J. List, R. Reiter, E. Morgan, M. B. Martin, and A. Stoica.** 2003. Estradiol rapidly activates Akt via the ErbB2 signaling pathway. *Mol Endocrinol* **17**:818-30.
230. **Strom, A., J. Hartman, J. S. Foster, S. Kietz, J. Wimalasena, and J. A. Gustafsson.** 2004. Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. *Proc Natl Acad Sci U S A* **101**:1566-71.
231. **Szlosarek, P., K. A. Charles, and F. R. Balkwill.** 2006. Tumour necrosis factor-alpha as a tumour promoter. *Eur J Cancer* **42**:745-50.
232. **Tee, M. K., I. Rogatsky, C. Tzagarakis-Foster, A. Cvor, J. An, R. J. Christy, K. R. Yamamoto, and D. C. Leitman.** 2004. Estradiol and selective estrogen receptor modulators differentially regulate target genes with estrogen receptors alpha and beta. *Mol Biol Cell* **15**:1262-72.
233. **Thomas, R. S., N. Sarwar, F. Phoenix, R. C. Coombes, and S. Ali.** 2008. Phosphorylation at serines 104 and 106 by Erk1/2 MAPK is important for estrogen receptor-alpha activity. *J Mol Endocrinol* **40**:173-84.
234. **Tonetti, D. A., R. Rubenstein, M. DeLeon, H. Zhao, S. G. Pappas, D. J. Bentrem, B. Chen, A. Constantinou, and V. Craig Jordan.** 2003. Stable transfection of an estrogen receptor beta cDNA isoform into MDA-MB-231 breast cancer cells. *J Steroid Biochem Mol Biol* **87**:47-55.
235. **Touillaud, M. S., A. C. Thiebaut, A. Fournier, M. Niravong, M. C. Boutron-Ruault, and F. Clavel-Chapelon.** 2007. Dietary lignan intake and postmenopausal breast cancer risk by estrogen and progesterone receptor status. *J Natl Cancer Inst* **99**:475-86.

236. **Tremblay, A., and V. Giguere.** 2001. Contribution of steroid receptor coactivator-1 and CREB binding protein in ligand-independent activity of estrogen receptor beta. *J Steroid Biochem Mol Biol* **77**:19-27.
237. **Tremblay, A., G. B. Tremblay, F. Labrie, and V. Giguere.** 1999. Ligand-independent recruitment of SRC-1 to estrogen receptor beta through phosphorylation of activation function AF-1. *Mol Cell* **3**:513-9.
238. **van de Wetering, M., E. Sancho, C. Verweij, W. de Lau, I. Oving, A. Hurlstone, K. van der Horn, E. Batlle, D. Coudreuse, A. P. Haramis, M. Tjon-Pon-Fong, P. Moerer, M. van den Born, G. Soete, S. Pals, M. Eilers, R. Medema, and H. Clevers.** 2002. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **111**:241-50.
239. **van Schaik, R. H., M. Kok, F. C. Sweep, M. van Vliet, M. van Fessem, M. E. Meijer-van Gelder, C. Seynaeve, J. Lindemans, J. Wesseling, L. J. Van 't Veer, P. N. Span, H. van Laarhoven, S. Sleijfer, J. A. Foekens, S. C. Linn, and E. M. Berns.** 2011. The CYP2C19*2 genotype predicts tamoxifen treatment outcome in advanced breast cancer patients. *Pharmacogenomics* **12**:1137-46.
240. **Watkins, R. E., G. B. Wisely, L. B. Moore, J. L. Collins, M. H. Lambert, S. P. Williams, T. M. Willson, S. A. Kliewer, and M. R. Redinbo.** 2001. The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. *Science* **292**:2329-33.
241. **Watson, A. J., and P. D. Collins.** 2011. Colon cancer: a civilization disorder. *Dig Dis* **29**:222-8.
242. **Vavouri, T., G. K. McEwen, A. Woolfe, W. R. Gilks, and G. Elgar.** 2006. Defining a genomic radius for long-range enhancer action: duplicated conserved non-coding elements hold the key. *Trends Genet* **22**:5-10.
243. **Weige, C. C., K. F. Allred, and C. D. Allred.** 2009. Estradiol alters cell growth in nonmalignant colonocytes and reduces the formation of preneoplastic lesions in the colon. *Cancer Res* **69**:9118-24.
244. **Weigel, N. L., and Y. Zhang.** 1998. Ligand-independent activation of steroid hormone receptors. *J Mol Med (Berl)* **76**:469-79.
245. **Weihua, Z., S. Andersson, G. Cheng, E. R. Simpson, M. Warner, and J. A. Gustafsson.** 2003. Update on estrogen signaling. *FEBS Lett* **546**:17-24.
246. **Wen, Y. D., V. Perissi, L. M. Staszewski, W. M. Yang, A. Krones, C. K. Glass, M. G. Rosenfeld, and E. Seto.** 2000. The histone deacetylase-3 complex contains nuclear receptor corepressors. *Proc Natl Acad Sci U S A* **97**:7202-7.
247. **Westerberg, H., S. A. Gustafson, B. Nordenskjold, C. Silfversward, and A. Wallgren.** 1980. Estrogen receptor level and other factors in early recurrence of breast cancer. *Int J Cancer* **26**:429-33.
248. **Wickramasinghe, N. S., T. T. Manavalan, S. M. Dougherty, K. A. Riggs, Y. Li, and C. M. Klinge.** 2009. Estradiol downregulates miR-21 expression and increases miR-21 target gene expression in MCF-7 breast cancer cells. *Nucleic Acids Res* **37**:2584-95.
249. **Wightman, B., I. Ha, and G. Ruvkun.** 1993. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell* **75**:855-62.
250. **Wijnhoven, B. P., M. Z. Michael, and D. I. Watson.** 2007. MicroRNAs and cancer. *Br J Surg* **94**:23-30.
251. **Williams, C., K. Edvardsson, S. A. Lewandowski, A. Strom, and J. A. Gustafsson.** 2008. A genome-wide study of the repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells. *Oncogene* **27**:1019-32.
252. **Vivar, O. I., X. Zhao, E. F. Saunier, C. Griffin, O. S. Mayba, M. Tagliaferri, I. Cohen, T. P. Speed, and D. C. Leitman.** 2010. Estrogen receptor [beta] binds to and regulates three distinct classes of target genes. *J Biol Chem*.
253. **Vo, N., and R. H. Goodman.** 2001. CREB-binding protein and p300 in transcriptional regulation. *J Biol Chem* **276**:13505-8.

254. **Wong, C. W., B. Komm, and B. J. Cheskis.** 2001. Structure-function evaluation of ER alpha and beta interplay with SRC family coactivators. ER selective ligands. *Biochemistry* **40**:6756-65.
255. **Wong, N. A., R. D. Malcomson, D. I. Jodrell, N. P. Groome, D. J. Harrison, and P. T. Saunders.** 2005. ERbeta isoform expression in colorectal carcinoma: an in vivo and in vitro study of clinicopathological and molecular correlates. *J Pathol* **207**:53-60.
256. **Vousden, K. H., and C. Prives.** 2009. Blinded by the Light: The Growing Complexity of p53. *Cell* **137**:413-31.
257. **Wu, W. K., P. T. Law, C. W. Lee, C. H. Cho, D. Fan, K. Wu, J. Yu, and J. J. Sung.** 2011. MicroRNA in colorectal cancer: from benchtop to bedside. *Carcinogenesis* **32**:247-53.
258. **Xie, D., J. S. Sham, W. F. Zeng, H. L. Lin, J. Bi, L. H. Che, L. Hu, Y. X. Zeng, and X. Y. Guan.** 2005. Correlation of AIB1 overexpression with advanced clinical stage of human colorectal carcinoma. *Hum Pathol* **36**:777-83.
259. **Xu, H. B., Y. J. Liu, and L. Li.** 2011. Aromatase inhibitor versus tamoxifen in postmenopausal woman with advanced breast cancer: a literature-based meta-analysis. *Clin Breast Cancer* **11**:246-51.
260. **Xu, H. E., M. H. Lambert, V. G. Montana, D. J. Parks, S. G. Blanchard, P. J. Brown, D. D. Sternbach, J. M. Lehmann, G. B. Wisely, T. M. Willson, S. A. Kliewer, and M. V. Milburn.** 1999. Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Mol Cell* **3**:397-403.
261. **Xu, L., C. K. Glass, and M. G. Rosenfeld.** 1999. Coactivator and corepressor complexes in nuclear receptor function. *Curr Opin Genet Dev* **9**:140-7.
262. **Zhao, C., H. Gao, Y. Liu, Z. Papoutsis, S. Jaffrey, J. A. Gustafsson, and K. Dahlman-Wright.** 2010. Genome-wide mapping of estrogen receptor-beta-binding regions reveals extensive cross-talk with transcription factor activator protein-1. *Cancer Res* **70**:5174-83.
263. **Zhao, J. J., J. Lin, H. Yang, W. Kong, L. He, X. Ma, D. Coppola, and J. Q. Cheng.** 2008. MicroRNA-221/222 negatively regulates estrogen receptor alpha and is associated with tamoxifen resistance in breast cancer. *J Biol Chem* **283**:31079-86.